THE BIOLOGY OF
PNEUMOCOCCUS

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THE BACTERIOLOGICAL, BIOCHEMICAL, AND IMMUNOLOGICAL CHARACTERS AND ACTIVITIES OF DIPLOCOCCUS PNEUMONIAE

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with the collaboration of

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Affectionately dedicated to the memory of

GEORGE HOYT BIGELOW, M.D.

*who gave to the Massachusetts Pneumonia Study
and Service an inspiring guidance, and who, by
his gallantry, set us an enduring and ennobling
example of scientific fellowship*

FOREWORD

IN the introduction to one of his *Lectures and Lay Sermons*, Huxley used an analogy aptly illustrating his subject, and singularly fitting the purposes of the present undertaking: "Merchants occasionally go through a wholesome, though troublesome and not always satisfactory, process which they term 'taking stock.' After all the excitement of speculation, the pleasure of gain, and the pain of loss, the trader makes up his mind to face facts and to learn the exact quantity and quality of his solid and reliable possessions. The man of science does well sometimes to imitate this procedure; and, forgetting for the time the importance of his own small winnings, to re-examine the common stock in trade, so that he may make sure how far the store of the bullion in the cellar—on the faith of whose existence so much paper has been circulating—is really the solid gold of truth."

The present undertaking is not an inventory of small personal winnings, but rather a re-examination of the circulating paper and an attempted appraisal of the quantity and quality of the stored bullion, done with a sense of humility before the importance and magnitude of the task. Nor is this an original enterprise. There are the familiar Rockefeller Institute *Monograph, Number Seven*, the ample summary of Neufeld and Schnitzer, and the later symposium in the British *System of Bacteriology*, but since their publication more gold has been mined, vastly enriching the common stock. In drawing upon this stock for the purpose of learning better ways of applying our knowledge to the discovery of means for mitigating the ills inflicted upon man by Pneumococcus, the whole storehouse has been ransacked for hidden or forgotten goods. To sort out the accumulation of more than fifty years, to convert old specie into modern currency, and finally to set a value on the whole store has been a troublesome task which it is hoped may be

spared others by the presentation of this re-examination and appraisal.

The present review of the literature on the biology of Pneumococcus forms a part of the Pneumonia Study and Service carried on during the years 1931 to 1935 by the Massachusetts Department of Public Health under a grant from the Commonwealth Fund.

The labor of preparing the manuscript has been lightened and made an agreeable occupation by the help so graciously given by many friends. An added pleasure, now that the work has come to completion, is to record grateful acknowledgments to the officers and members of the staff of the Commonwealth Fund for their sympathetic interest and liberal support; to Professor Doctor Fr. Neufeld for permission to reproduce original drawings from his publications and for aid in securing some of the portraits included in the text; to Dr. Rufus Cole and colleagues at the Hospital of the Rockefeller Institute for Medical Research for many courtesies; to Dr. O. T. Avery for the rich benefits of his counsel; to Dr. M. A. Dawson for the generous loan of unpublished illustrative material; to Miss L. M. D. Trask, librarian of the Rockefeller Institute, for her able assistance in searching the literature; to Miss E. C. Campbell of the Publication Division of the same Institute for the loan of original electrotypes from the *Journal of Experimental Medicine;* to Miss Emily Jackson and associates at the Massachusetts Antitoxin and Vaccine Laboratory for their competent and willing services; to many of the authors quoted for criticism and suggestions; and to Mrs. Helen M. Boynton for her intelligent and discriminating digest of the whole story of Pneumococcus.

B. W.

Boston, 1937

CONTENTS

stituent of the pneumococcal cell. Cause of differences in
carbohydrates isolated from Pneumococcus. Relation of
the acetyl group to the immunological activity of Pneumo-
coccus. Recent methods of preparing capsular polysaccha-
ride. Other physicochemical properties of the capsular
polysaccharide. Summary.

INTRODUCTION

Pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death. Furthermore, living or dead, whole or in part, on entering the animal body Pneumococcus starts a train of impulses, stimulating all the reactions grouped under those inclusive phenomena known as immunity.

Digesting foreign proteins, Pneumococcus rebuilds the fragments into a new protein common to all types of the species; splitting and consuming carbohydrates, from the simplest sugars to the starch-like substances, inulin and glycogen, the cell synthesizes the cleavage products of these same sugars into complex polysaccharides. These polysaccharides, chemically distinct and immunologically specific for the type, are built into a morphological structure which forms a defensive armor against the destructive forces of the animal body, and in a highly selective fashion determines the precise nature of the immunological response each separate type calls forth.

Man has not been content to allow Pneumococcus to destroy human life in an unrestrained way. The sanitarian has sought hygienic and prophylactic measures to curb the incidence of acute respiratory infections; the pathologist has studied the anatomical distribution and histological nature of pulmonary lesions in order to gain fuller knowledge of the portal of entry and the pathway of infection through the living tissues; the epidemiologist has attempted to track the mode of dissemination of the infectious agent in the hope of breaking the vicious cycle of transmission from man to man; pending the discovery of specific therapy, the physician has ameliorated suffering by symptomatic treatment and con-

tributed to recovery by instituting supportive measures until the
natural capabilities of living tissue have repaired the injuries in-
flicted by the invading microorganisms.

The bacteriologist has persisted in the pursuit of information,
and as a result the positive identification, the growth needs, and
much of the physiological behavior of Pneumococcus are now com-
mon knowledge. The immunologist, by serum reactions, has found
that pneumococci are not all alike; that they may become de-
graded and even transmuted into strange forms, and that on this
degradation or exaltation depend certain vital processes within
the cell related to virulence and the morbid effects produced in the
animal economy. With this information he has devised ways of
identifying types among the species, and of using the cocci for
his own purposes.

In time the chemist joined forces with his colleagues in the field
of medical science, and as a result of their combined efforts the
answer has been found to some of the riddles which the bacteri-
ologist alone could not solve. Breaking down Pneumococcus into
its component parts, the chemist found the expected protein, and
then discovered the new sugars which have so altered our ideas
about pneumococci in general, and which are bringing a solution
to the cryptic working of the immunological mechanism.

The chemist, laboring with the bacteriologist and immunologist,
has given us a new conception of the significance of chemical con-
stitution and molecular arrangement in determining the power of
proteins and of carbohydrates, either alone or combined, to call
into activity those reactions which spell the fate of the infected
host or the fate, not only of Pneumococcus, but of other bacteria
and even of non-living alien substances that gain entrance to the
animal body. New biological principles have been disclosed, ex-
plaining many of the functions of the living bacterial cell amidst
both natural and artificial surroundings and opening fresh and
inviting paths of investigation.

The study of the members of this small group of microorganisms

in a subordinate branch of biology is bringing light into some of the obscure realms of the related sciences. The peculiarities of Pneumococcus are yielding a generous return to the investors and speculators who have cast in their resources with its lot, resulting in the accumulaticn of a store of solid bullion for the scientist and for mankind.

THE BIOLOGY OF
PNEUMOCOCCUS

LOUIS PASTEUR 1822-1895

By Louis Edouard Fournier, École Normale Supérieure, Paris

HISTORY OF PNEUMOCOCCUS

A chronological account of the early observations of the coccus in tissues, saliva, and other body fluids; first isolation and cultivation, and description of effects on animals; separation into types; variation; and attempts to produce immune serum.

THE lanceolate, Gram-positive diplococcus, now known as the chief etiological agent in lobar pneumonia and commonly called Pneumococcus, has the species name *Diplococcus pneumoniae*, genus *Diplococcaceae*, tribe *Streptococcaceae* of the family *Coccaceae*.[104] During the years in which the organism has been the subject of investigation, it has received many appellations which are given below in chronological order.

Monas pulmonale (Klebs)[718]	1875
Microbe septicémique du saliva (Pasteur)[1066]	1881
Pneumoniekokken (Mátray)[869-70]	1883
Coccus lancéolé de la pneumonie (Talamon)[1377]	1883
Pneumoniemikrokokken (Friedländer)[487]	1883
Pneumonie-Micrococcen (Friedländer)[490]	1884
Micrococcus Pasteuri (Sternberg)[1319]	1885
Pneumoniemikrococcus (Fraenkel)[469]	1886
Pneumococcus (Fraenkel)[469]	1886
Bacillus septicus sputigenus (Flügge)[495]	1886
Diplokokkus lanceolatus pneumoniae (Flügge)[495]	1886
Diplococcus pneumoniae (Weichselbaum)[1497]	1886
Bacillus salivarius septicus (Biondi)[117]	1887
Micrococcus pneumoniae crouposae (Sternberg)[1320]	1887
Streptococcus lanceolatus Pasteuri (Gamaléia)[498]	1888
Diplococcus lanceolatus (Foà, Bordoni-Uffreduzzi)[462]	1888
Virus pneumonico (Gabbi)[497]	1889
Bacterium pneumoniae (Migula)[901]	1900
Diplococcus pneumoniae Weichselbaum (Bergey)[104]	1930

In giving proper credit for the discovery of an organism it usu-

ally becomes necessary to decide which special events constitute a discovery. Is it the report of the first sight of the organism in the fluids or tissues of the infected animal; is it the first formal association of the virus with a disease; is it the setting up of a specific infection in an alien animal species; or must one await the complete fulfilment of all the postulates of Koch's law?

It is not essential to our purpose to be too exacting in asking or answering these questions. It should suffice to describe the observations of early pioneers and their successors in the order of their happening.

1875–1890

In 1875, Klebs[718] was probably the first to see—or at least the first to tell about seeing—Pneumococcus. Searching for proof of the infectious nature of pneumonia, he examined fluid from the lungs of men dying of the disease and found non-motile, sometimes linked monads in astonishing numbers. Growing these cocci on egg albumen, he thought they developed motility when grouped in chains, but their true identity escaped him. In 1880, Eberth[347] was undoubtedly looking at pneumococci when he examined the exudate from a gray-hepatized lung and the subarachnoid fluid of a pneumonia patient with a secondary meningitis. His description of the organisms as non-motile, slightly oval, almost round bodies, occasionally occurring singly but more often in pairs, taken with their source, makes it seem more than likely they were pneumococci, but Eberth thought they were only varieties of diphtheria or pyemia micrococci. Mátray,[869-70] in the same year, gave the name *Pneumoniekokken* to the cocci which he found in the sputum of pneumonia patients and in normal sputum as well. Had animal inoculation been tried by either Eberth or Mátray their find would have preceded the real discovery by Pasteur and Sternberg by over a year; but it was not tried.

In 1881, these two authors, Pasteur and Sternberg, independently inoculating rabbits, the former with the saliva of a child

dead from rabies, the latter with normal saliva, isolated Pneumococcus for the first time through animal passage. Pasteur, contrary to the subsequent opinions of others, thought that he had discovered a new disease.* Sternberg reported that the fatal septicemia which normal saliva produced in rabbits was due to an organism that later, upon microscopic examination, appeared to agree exactly with the description given by Pasteur.

Pasteur[1065-6] passed his virus from infected to normal rabbits, producing infection each time, recovered the organism from the blood by cultivation in bouillon and other media, and with these cultures induced a similar fatal septicemia in other rabbits. On microscopic examination he found the bacteria to be very short rods, somewhat depressed in the center, with a visible aureola due, as he thought, to mucin.

Sternberg[1316-8] ascertained that the ability to incite fatal rabbit septicemia was a peculiarity of the saliva of some normal individuals but not of others, and that normal blood, putrid urine, liquid feces, and bouillon undergoing spontaneous putrefaction failed to produce a similar infection. In a later study appearing after the publication of Pasteur's paper, using aniline violet as a stain and making a permanent record by photomicrographs, Sternberg confirmed Pasteur's and his own findings. Here the dates are worth noting. Pasteur announced his discovery before the French Academy of Sciences in December, 1880, the report appearing in the Academy's *Comptes Rendus* in January, 1881. Sternberg began his experiments in the summer of 1880, and made his report which was first published in April, 1881.

There then arose a misconception of Pasteur's claims which was to persist for a long time. By his fellow countryman, Colin,[269] he was gratuitously credited with the claim that he had isolated the microbe of rabies. Pasteur emphatically replied, *J'ignore absolument les relations de cette nouvelle maladie avec la rage.* Pasteur

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* The original communication was presented by Pasteur, but the work was done with the collaboration of Chamberland and Roux.

also stated that this new disease was not a septicemia, and this mistake undoubtedly contributed to the confusion of ideas. Disturbed by this false imputation, Pasteur, in March, 1881, in a letter to Parrot[1064] who had, like Sternberg, produced a fatal infection in rabbits with normal saliva, again stated positively that his organism bore no relation to rabies. Pasteur, by the way, showed his great perspicacity in a paragraph which was included in that letter. It bears repeating: *J'y vois, pour ma part, un symptôme nouveau de grand avenir pour la connaissance étiologique des maladies dont la cause doit être attribuée à la présence et au développement d'organismes microscopiques.* Again, in a communication read at the May 31st session of the *Académie de Médecine*, Pasteur sharply protested against the claims ascribed to him.

In this connection, the originals of Pasteur's and of Sternberg's later papers (1885 and 1887)[1319-20] are well-worth reading. In these days of petty priority claims it is refreshing to read of these two gentlemen of the eighteen-eighties, of whom one, Pasteur, frankly acknowledged his ignorance, while the other granted precedence and magnanimously named the organism after Pasteur. Sternberg's statement (1885) ran as follows:

> In attaching to this micrococcus the name of the illustrious French chemist I have no desire to perpetuate the memory of the mistake he made in supposing for a time that it was the germ of hydrophobia. Having found that this was a mistake, he did not fail to correct it. . . . It is easy to make mistakes in this field of investigation; easier, perhaps, than to acknowledge them.* And believing, as I do, in human fallibility, I have no hesitation in questioning the conclusions of the most illustrious workers in the field of microbiology, if they are in conflict with my own observations. On the other hand, if, upon fuller investigation, I am convinced that I have been mistaken in regard to this or any other question, I shall feel no hesitation in following the example of Pasteur in making a public announcement of my error.

Here is material for a Hippocratic oath for the biologist or any other fellow of science.

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* Sternberg was prominent among those who had given an entirely erroneous interpretation to Pasteur's claims, and this statement, gracious though it was, served to foster the unfortunate misconception.

Vulpian,[1453] at the March (1881) meeting of the French Academy, said that he regretted that he had not been present at a former session when the letter from Pasteur to Parrot had been read, because he also might have reported that he had caused the death of a rabbit by the subcutaneous injection of normal saliva, while the blood of that animal killed another rabbit within the space of two days. The blood of the animals contained a number of "microbes," the majority having the same characteristics as those described by Pasteur. Here was the link between the work of Pasteur and that of Sternberg, but Vulpian, besides failing to appreciate the connection, was tardy in his announcement. Claxton (1882),[237] too, found normal human saliva infectious for rabbits. Believing in Pasteur's idea, he however confirmed Sternberg's results, with the added observation that human saliva varied in its infecting power, that of individuals from such tropical countries as Cuba and Brazil being extremely virulent.

In 1881, Osler[1039] spoke of finding "micrococcus balls" in four cases of endocarditis complicating pneumonia. He was not willing to assert they were the cause of the disease, but called attention to the frequency with which this condition and meningitis accompany pneumonia. Osler's comment recalls the earlier description by Koester (1878)[736] of "bullet-like masses of micrococci" in embolic endocarditis, which may or may not have been pneumococci.

The year 1881 was an eventful one. If one examines the photomicrographs accompanying Robert Koch's[735] paper on pathogenic organisms, there is little difficulty in identifying these lance-shaped diplococci, but to Koch they were that and nothing more. Poincaré[1102] was nearer the truth when he spoke of finding "prodigious numbers of little spheres of double contour" in pneumonic lung tissue, but he lacked the support of positive animal experiments to justify a claim that these organisms were responsible for pneumonia.

Shortly after the preliminary announcements of Pasteur and of Sternberg, Friedländer (1882)[486] presented a communication which takes high rank in the history of Pneumococcus. He reported that

in September, 1881, in the fibrinous exudate and in hardened sec-
tions of lung and pleural tissue, stained by the Weigert-Koch
method, from eight cases of acute pneumonia and in the fluid with-
drawn from living pneumonia patients by lung puncture, he had
observed spherical and ellipsoidal cocci, occurring mostly in pairs,
and sometimes in longer chains made up of diplococci.

In 1882, Günther,[579] at a meeting of the *Verein für innere Medi-
zin* in Berlin, had reported that the microscopic examination of
stained preparations of purulent, bloody matter from lung punc-
ture showed numerous diplococci. He gave a demonstration of his
preparation and exhibited a drawing which caused Fraenkel to re-
mark that the cocci seemed to be surrounded by an unstained rim.
Günther replied that the rim was really a hull. Early in the same
year, Colomiatti,[271] who was familiar only with the work of Klebs,
reported finding *Monas pulmonale* in a growth on the heart valve
and in the subarachnoid fluid in a case of croupous pneumonia
complicated with endocarditis and meningitis.

In 1883,* Friedländer[487-8] announced that he had found the same
micrococci in the alveolar exudate in all but a few of fifty cases
of pneumonia. Stained by the method of his colleague, Gram,
these cocci took an intense color, which was not extracted by treat-
ment with alcohol, and exhibited a well-defined capsule. The cap-
sule, which a short time before had been observed by Günther,
could be clearly demonstrated by fixing the preparations with
acetic or mineral acids and staining with gentian violet, fuchsin,
Bismarck brown, or methylene blue. The capsular substance was
insoluble in alcohol, ether, or chloroform, and was best shown by
preliminary staining with aniline gentian violet, a short exposure
to alcohol, and counterstaining with eosin. The contour was
brought out by osmic acid but there was no blackening of the cap-
sule or cell. Friedländer observed that the capsules were most char-
acteristic at the highest stage of the cells' development, and con-

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* A greater part of this work was done with the collaboration of Frobenius,
who, because he did not remain until the completion of the study, declined the
inclusion of his name in the authorship of the paper!

sidered them not as a passive precipitate, but as a product of the
vital action of the coccus. Applying several microchemical tests to
the capsular material, he felt justified in concluding that it con-
sisted of mucin or an allied substance. When one remembers that
mucin is a protein-carbohydrate complex in which the saccharide
yields on hydrolysis two glucosamin, one acetic acid, and two
hexose molecules, one appreciates the value of this observation in
anticipating the discovery of the soluble specific substance or cap-
sular polysaccharide by Heidelberger and Avery. Friedländer cul-
tivated these organisms on Koch's coagulated blood serum, upon
which they grew as round and elliptical cocci. His descriptions of
the colonies on this medium corresponded with the appearance of
the colonies of typical pneumococci. He also said that he had car-
ried the cultures through eight transfers in meat-infusion peptone
gelatin. In this medium the coccus lost its capsule and grew in
"nail-form" colonies.

When one-half to one cubic centimeter of aqueous suspensions of
the gelatin cultures was injected into the lungs of rabbits no in-
fection resulted. Guinea pigs inoculated in this way were more sus-
ceptible, six of nine becoming infected. Mice were far more sus-
ceptible, all dying of infection. From the pleural and lung exudate
of the infected animals Friedländer isolated typical diplococci. In
four of twelve mice he succeeded in producing infection by inhala-
tion. One of four dogs succumbed to the injection of cultures into
the lung, and this animal at necropsy showed red and gray hepa-
tization of the lung. From these areas, from the blood, and from
the right pleural cavity, Friedländer recovered typical organisms.
He noted variations in the capsule of the organisms depending
upon their propagation in mice, guinea pigs, dogs, and man.

At that time, Friedländer was unable to demonstrate cocci in the
blood of pneumonia patients, but in 1884[489-90] reported that he
had cultivated typical micrococci from the blood of one of six pa-
tients. The cocci were encapsulated and virulent for mice, but by
the method tested were avirulent for rabbits. It was in his first

communication that, after giving credit to Klebs for the first ref-
erence to Pneumococcus and to Eberth and Koch for previous re-
ports, Friedländer gave the name *Pneumoniemikrokokken* to the
organisms. He believed that the capsule and the nail-form colony
were not distinctive diagnostic characters, but that the whole cycle
of phenomena must be observed, namely, the effect on animals and
recultivation of the cocci from the artificially infected animals. He
agreed that it was impossible to obtain this complete series of
events in all cases of pneumonia, and suggested that there might be
several forms of pneumonia, one of which was caused by the *Pneu-
moniemikrokokken*, or that the organisms while present at one
stage of the disease were either not present or dead at other stages.
He inclined to the first viewpoint.

Salvioli and Zäslein (1883)[1217] examined the sputum, canthari-
des blister fluid, and the blood of pneumonia patients. Here the
same cocci, less frequent in the sputum than in the other fluids,
could be cultivated in broth and in Pasteur's solution and ren-
dered visible with Bismarck brown and methyl violet.

In 1883, Strassmann[1343] also found diplococci in pneumonic
sputum.

In the same year, before the Berlin Medical Society, von Ley-
den demonstrated in blood and exudate aspirated from the hepa-
tized lung of a pneumonia patient diplococci, largely oval in form,
occasionally in longer chains. He added that similar cocci could be
seen, without staining, in small masses scraped from the affected
lung at necropsy. He surmised that Klebs had seen the same or-
ganisms.

Considering the meagerness of information in his time (1882),
Bozzolo[145] contributed some sound ideas. He concluded from his
studies that pleurisy, pericarditis, endocarditis, and cerebrospinal
meningitis were frequently associated with lobar pneumonia; that
this association, whether the disease occurred in epidemic or spo-
radic form, was caused by the action of a single infecting agent;

GEORGE MILLER STERNBERG 1838-1915
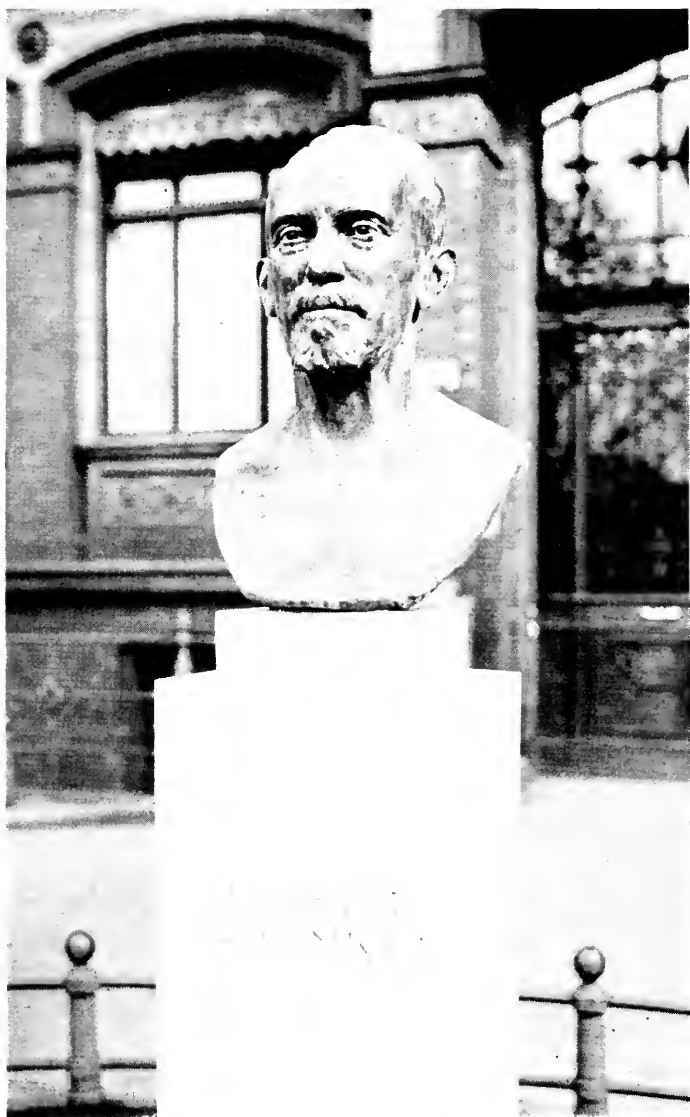
ALBERT FRAENKEL 1848-1916

By Alexander Oppler, Städtisches Krankenhaus, Berlin

and that the agent, as demonstrated by sufficient scientific facts, could be said to be a species of *Schyzomycetes*.

Ziehl,[1574] familiar with the work of Klebs, Eberth, Friedländer, and von Leyden, sought bacteria in the sputum of two pneumonia patients. He saw ellipsoidal cocci in the early stages of the disease, and the same organism mixed with many other bacterial forms in the later stages. Peiper[1076] reported the presence of large numbers of diplococci in lung-puncture fluid. That primary sporadic cerebrospinal meningitis could be caused by Pneumococcus was first discovered by von Leyden[810] but, because the oval diplococci appeared to be slightly larger than those found in pneumonia, he was in doubt as to their true nature.

Late in 1883, Talamon,[1377] although lacking conclusive experimental data, felt justified in stating that lobar, fibrinous pneumonia was an infectious disease produced by a specific microbe with a characteristic form, and he named the organism, *Coccus lancéolé de la pneumonie*. He found this coccus in the pulmonary exudate taken by lung puncture, and once in the blood during life. Since only a fluid medium was used, the cultures were seldom pure. Talamon failed to infect guinea pigs or dogs with this mixed growth, but sixteen out of twenty rabbits succumbed to inoculation, although none developed pleuritis, pericarditis, or pneumonia. Notwithstanding the fact that his experimental results did not fulfill all the requirements of Koch's law, the claim was correct, and the statement that pneumonia is a localized disease, principally pulmonary, and susceptible of becoming generalized in other organs, was true.*

During the year 1884, interest in Pneumococcus spread and fresh information came from various sources. Gram[545] himself gave the details of the indispensable stain which now bears his name, and described how the capsule first took the dye and then gave it

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* Mendelsohn (1884)[892] gave an excellent review of the current ideas on the epidemiology and infectiousness of pneumonia.

up to alcohol, while the coccus retained the violet color. Then Em-
merich,[355] after hunting bacteria in air, water, dust, and dirt, re-
covered encapsulated diplococci from the filling between the floors
of a prison in Augsburg, notorious for over twenty-five years as a
breeding place of pneumonia. He cultivated the cocci on meat-
infusion peptone gelatin, separated them from accompanying bac-
teria, injected the purified culture into rabbits with indifferent suc-
cess, but obtained a higher fatality rate when he used mice. He
identified the coccus as that described by Friedländer, and gave
to the report the title, *Die Auffindung von Pneumoniecoccen.* . . .
Emmerich's discovery, confirmed several decades later by the work
of Stillman,[1326] furnished one of the first epidemiological clues to
the origin of some cases of pneumonia.

Somewhat analogous to Emmerich's discovery of pneumococci in
the floor filth of a pneumonia-infected prison was the apparent
success of Pawlowsky (1885)[1072] in isolating, on solid media, Pneu-
mococcus-like organisms from the air of various rooms. Influenced
by the teaching of Friedländer, Pawlowsky, because his cocci were
slightly smaller (they were also associated with larger cocci) and
because they were pathogenic for rabbits, was not sure of his
ground. The infectiousness of these cocci for rabbits, guinea pigs,
and dogs indicates that among the bacteria described by Paw-
lowsky there were pneumococci. Cornil and Babes[278] added a note
to the effect that they had found lancet-shaped bacteria in the ton-
sils and from the endocardium of pneumonia patients, but they
made no cultures.

Exudates from the pleura and pericardium of two pneumonia
patients yielded, in Salvioli's[1215] hands, encapsulated cocci which,
while fatally infective for guinea pigs, rabbits, and dogs, failed to
cause the typical lesions of pneumonia. Thinking that he had over-
whelmed the animals with the virulence of the fluids injected, he in-
troduced the infectious material intratracheally into four guinea
pigs, two of which died. Platonow,[1095] in a detailed and critical re-
view of the existing literature, concluded from the evidence and his

own experimental experience that neither the capsule nor the nail-form colony was a differential character and that bacteriological examination of the blood of pneumonia patients was not of diagnostic value. From Platonow's notes it would seem that he used mixed cultures of bacilli and cocci, so his doubts were well founded.

In April of that year (1884), Fraenkel[466] presented the first of a series of studies that were to make the name Fraenkel and Pneumococcus almost inseparable. In a discussion of the cause of pneumonia before the *Kongress für innere Medizin* in Berlin, Fraenkel displayed a human trait that at least enlivens the sober literature if it does not always bring the desired personal reward. Fraenkel complained that he should receive some of the credit for the discovery of Pneumococcus. He had begun experiments six months before Friedländer's report was made public, and had only delayed the announcement because his results differed from Friedländer's and because he wished to recheck them! He had scored on his fellow countryman by infecting rabbits with the coccus where Friedländer failed, but his further observations had not been so positive. There was a difference in the pathogenicity of some of the materials which he used; colony appearance and capsule formation were not constant characters and, moreover, other bacterial species had capsules. Apparently unaware of Sternberg's experiments, Fraenkel[468] had missed the meaning of his success in producing a fatal septicemia in rabbits with normal sputum and the subsequent recovery by cultivation on coagulated blood serum of encapsulated diplococci from the blood of the animals. He had hesitated to draw any comparison between the diplococci from pneumonic material and his "sputum septicemia coccus." Likewise, he had ventured no outright statement that his and Friedländer's organisms were separate species; in fact, he gave the impression that they were different forms of the same organism. Friedländer, in the discussion of Fraenkel's paper, suggested that there might be several organisms causing pneumonia, so he, for the while, had the last word.

Here began a controversy, fomented principally by Fraenkel,

despite the conciliatory attitude of Friedländer, which led to confused conceptions which endure to the present day and which can never be clarified. Fraenkel, in a paper published in 1885,[467] virtually acknowledged that the coccus described by Friedländer and the one described by himself were identical, but complained that he saw it first. It has long been the verdict that the organism described by Friedländer in 1882 and 1883 was not Pneumococcus but the bacillus which later came to bear his name. This verdict would deprive Friedländer of any credit for the original isolation of Pneumococcus from lobar pneumonia and bestow it on Fraenkel who, without it, still has the honor of giving the first complete descriptions of Pneumococcus and whose studies proved for the first time the etiological relationship between this coccus and lobar pneumonia in man.

The facts given in Friedländer's first and second papers admit of more than one interpretation. The organism which Friedländer first described was an elliptical, or round, encapsulated coccus occurring principally in pairs. It was isolated from nearly all of fifty cases of acute pneumonia and was Gram-positive. It need scarcely be pointed out that these are characters of Pneumococcus and not of *B. friedländeri*.* The culture was exquisitely virulent for mice. However, the lack of pathogenicity for rabbits, its ability to grow on gelatin at room temperature with the formation of nail-like colonies are not characteristic of typical pneumococci. Perhaps Friedländer, in the face of strong opposition, weakened his position by acknowledging that the cycle of isolation of the coccus from man, the production of infection in animals, and its subsequent recovery could not be accomplished in every case of pneumonia. Possibly another factor tending to detract from the true import of his discovery may have been his suggestion that there might be different forms of Pneumococcus, or that pneu-

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* Inasmuch as the earlier name, *Bacillus friedländeri*, occurs in the great majority of the original publications reviewed, it, rather than the newer name, *Klebsiella pneumoniae*, will be used in this text.

monia might be caused by other organisms; ideas that we now know are true but which were scouted at the time. A careful reading of Friedländer's original communications makes it difficult to escape the conviction that he, in 1881, saw in the secretions and tissues of pneumonia patients the organism we know as Pneumococcus and, in the next two years, accomplished its isolation and cultivation. It seems likely, however, that Friedländer isolated at the same time the bacillus which later came to bear his name.

One seems justified in assuming as a cause of the divergent opinions which have so long persisted in the literature, the statement in Friedländer's communication of 1886[492] that the micrococcus so thoroughly studied by Fraenkel was the coccus he had originally described, but that the organism upon which he was then reporting was neither a coccus nor a bacillus* but a bacterium which he called *Kapselbacterium*. In addition to short elements, it existed in rod-like and thread-like forms and, furthermore, it was found only in a minority of the pneumonia cases studied—both characters of the Friedländer bacillus. Therefore, in view of all the evidence, and with due deference to the opinion of distinguished authorities, it would seem that the credit for first indicating that a diplococcus—*Diplococcus pneumoniae*—might be the cause of pneumonia should be given to Friedländer. To him, also, should go the honor of discovering another etiological agent of pneumonia, *Bacillus friedländeri*.

Afanassiew,[4] in 1884, added suggestive, if somewhat doubtful, information to the question. He, too, obtained ovoid cocci from six cases of pneumonia, but the cultures were not pure and this fact led to uncertainty. Klein,[719] on the contrary, apparently succeeded not only in growing pure cultures of Pneumococcus and in infecting mice and rabbits with them, but in recovering the organisms from the test animals and in transmitting the infection in series to

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* It should be borne in mind that, at the time, the criterion for judging whether an organism was a bacillus or a bacterium was the presence of motility in the former and its absence in the latter.

other rabbits and mice. The infection was a septicemia and never a localized process as claimed by Friedländer. Klein had no hesitation in using the name *Pneumokokkus,* in the title of his paper.

Maguire (1884),[855] in England, presented before the British Medical Association three preparations to illustrate the micrococcus of pneumonia. One was a section of a pneumonic lung, another a section of kidney from a case of pneumonia, and the third, sputum from a similar patient. Employing both methylene blue and the Gram stain, he found capsules on the cocci, although they were not always present. Maguire exhibited the specimens as illustrating Friedländer's micrococcus but, lacking cultural or inoculation experience, showed caution in saying that the question of the role of Pneumococcus must remain in abeyance until more data were forthcoming.

In the same year, Foà and Rattone,[464] working with a pure culture of Friedländer's "Pneumococcus" obtained from his colleague, Frobenius, duplicated Friedländer's observations even to the resistance of rabbits to its invasion. They confirmed the results with cultures isolated from the lung of a pneumonia patient, and were the first to succeed in producing meningeal infection in guinea pigs by inhalation, recovering "capsule-cocci" from the exudate on the pia mater.*

By 1885, Sternberg[1319] was ready to state that the pneumoniacoccus of Friedländer, the *Microbe septicémique du saliva* of Pasteur, and the organism he himself had isolated from normal saliva were not only the same but were probably the cause of pneumonia.†
His conclusion bears quoting: "It seems extremely probable that this micrococcus is concerned in the etiology of croupous pneumonia, and that the infectious nature of this disease is due to its presence in the fibrinous exudate into the pulmonary alveoli." Its

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* Jürgensen[699] reviewed the literature up to his time (1884) and concluded, "True pneumonia is an infectious disease, which principally but not exclusively, affects the lungs."

† Sternberg, after seeing the Friedländer culture in Koch's laboratory in 1885, changed his mind, and in 1887[1320] said he was in error in thinking that this and his saliva-coccus were identical. He then tentatively changed the name to *Micrococcus pneumoniae crouposae.*

presence in normal human saliva seemed to Sternberg to indicate that some other factor was necessary for the development of an attack of pneumonia. It was possible that some condition, such as "alcoholism, sewer-gas poisoning, crowd-poisoning or any other depressing agency" might render the individual vulnerable, while a "reflex vaso-motor paralysis, induced by cold, might affect a single lobe of the lung." It was at this time that Sternberg graciously gave the name *Micrococcus Pasteuri* to the coccus.

Fraenkel (1886),[468] now reassured by his experiments but still conservative, issued another note, preliminary to a more positive statement to appear later, to the effect that diplococci were sometimes, but not always, found in normal human saliva, more often in the saliva of a sick person, and still more frequently in the rusty sputum of pneumonia patients. He called the disease produced by this organism in rabbits and mice, "sputum septicemia." The organisms failed to grow on gelatin at room temperature, but on congealed blood serum or on agar at body-heat developed characteristic veil-like or dewdrop-like colonies. A similar coccus was present in two cases of empyema following pneumonia, but in some cases of empyema other organisms were to be found in the pus. Fraenkel then went so far as to say that these facts would make it seem highly probable that the microbe of sputum septicemia and Pneumococcus were identical.

In the following year (1886), more positive in his convictions, Fraenkel[469] was ready to grant that his coccus—that of sputum septicemia—the sputum coccus of Pasteur and that of Sternberg, as well as the organisms described by Talamon and by Salvioli, were identical, and then to state definitely that this coccus was the cause of true fibrinous pneumonia. With new assurance Fraenkel became somewhat caustic in his comment about Friedländer's claims and flatly avowed that his organism and that of Friedländer were not the same. He then named it *Pneumoniemikrococcus*, calling it also Pneumococcus. Here, more so than previously, Fraenkel's experiments are impressive for their care and thoroughness.

He grew the cocci on liquid and solid media, studied the most favorable reaction for growth, and found that while the organisms retained their virulence on coagulated blood serum or meat-infusion agar, they lost it rapidly in broth because of its changed reaction during incubation. Young cultures were highly pathogenic for mice and rabbits, less so for guinea pigs, and avirulent for dogs, pigeons, and chickens.

Fraenkel was able to isolate the diplococcus from all cases of true fibrinous pneumonia, grew it in pure culture, produced a fatal septicemia in susceptible animals, again recovered from them the organism in a pure state, and then further transmitted the infection to other test animals. His results therefore met all the requirements of Koch's law, and it would seem that Fraenkel was the first to establish the fact beyond a reasonable doubt that Pneumococcus was the causative agent in lobar pneumonia.

Fraenkel[468] made another important and original contribution when he reported that rabbits, recovering from a subcutaneous infection of the ear, resisted a subsequent inoculation with the same coccus. Although Foà and Bordoni-Uffreduzzi had independently discovered the same phenomenon (1884), this is probably the first controlled observation that pneumococcal infection may induce immunity.

Fraenkel also found Pneumococcus in the exudates in the pia mater from a case of meningitis accompanying pneumonia, but was unable to decide which lesion was primary. Senger (1886)[1255] had seen these diplococci in amazing numbers in fluid from the subarachnoid and ventricular spaces in similar cases and, in addition, in the lesions of endocarditis, pericarditis, pleuritis, and nephritis of pneumonia patients. He was, of course, correct in supposing that these and other metastases might be expected to arise from a pulmonary lesion. Netter,[962-3, 965] and also Lancereaux and Besançon,[778] reported the same findings in two corresponding cases of meningitis, pericarditis, and endocarditis. Lebashoff,[795] in Russia, at this time announced similar observations on the occurrence of
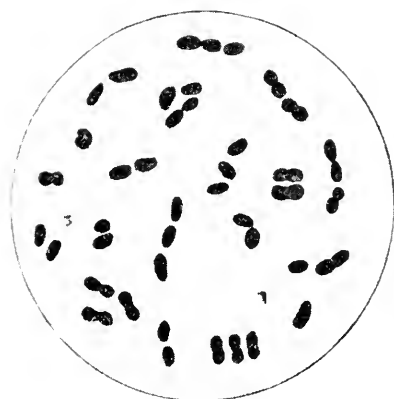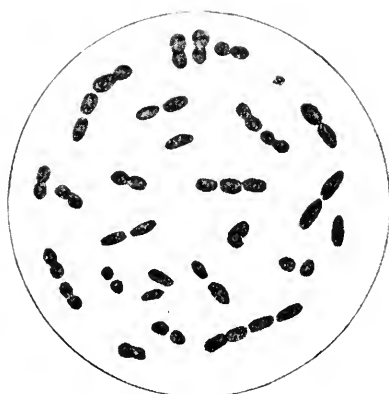
Figure 1   Figure 2
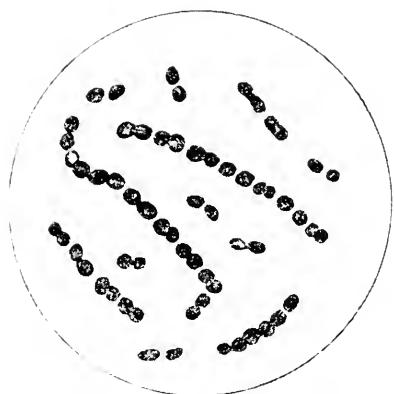
Figure 3   Figure 4
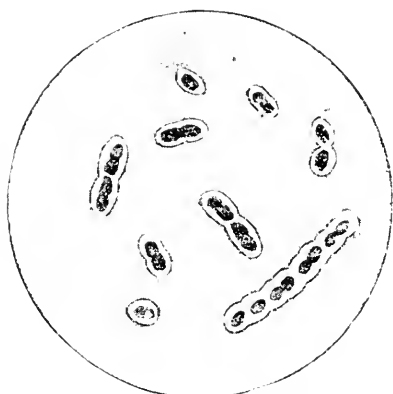
After Sternberg[339]

MICROCOCCUS PASTEURI, 1885

Figure 1. *Micrococcus Pasteuri* from blood of rabbit inoculated subcutaneously with normal human saliva (Dr. S.). Stained by the method recommended by Friedländer. Magnified 1000 diameters. Figure 2. *Micrococcus Pasteuri* from blood of rabbit inoculated subcutaneously with fresh pneumonic sputum from a patient in the seventh day of the disease. Same staining and amplification as Figure 1. Figure 3. Surface culture of *M. Pasteuri*, showing development of long chains. Same staining and amplification. Figure 4. Surface culture of *M. Pasteuri* from blood of rabbit injected with pneumonic sputum, showing the so-called "capsule" of Friedländer. Same staining and amplification.

these diplococci in other organs as well as in lung tissue but, evidently being ignorant of Fraenkel's work, he described the organisms as Friedländer's cocci.

In France, Gamaléia (1886),[498] after isolating, cultivating, and inoculating into several different species of animals, cocci obtained from lungs and fluids from meningitis and endocarditis complicating pneumonia, announced that the encapsulated lancet-form diplococcus derived from these sources was the same as the organism originally described by Pasteur. As a compliment to his chief, but deceived by the occasional chain formation, Gamaléia named it *Streptococcus lanceolatus Pasteuri*. In offering an explanation for the resistance of man to its invasion, he drew attention—an original observation—to the possible part played by leucocytes in the body's defense.

Pneumococcal infection of the kidneys was first reported by Nauwerck[944-6] in 1886. Among 550 cases of croupous pneumonia there were thirteen complicated with acute nephritis and in these instances he found in the kidneys cocci which he said were identical with those described by Friedländer. Nauwerck stated that the infection was specific and was caused by "pneumonia cocci" carried to the kidney by the blood.

At this time the differentiation began between Fraenkel's Pneumococcus and the bacillus of Friedländer. Weichselbaum, and Foà and Bordoni-Uffreduzzi sharply distinguished between the two organisms, although it may be again emphasized that Friedländer's first descriptions were those of a diplococcus and not of a bacillus. Some of the credit usually given to Fraenkel's contributions may be granted to Foà and Bordoni-Uffreduzzi[460] whose work was done independently of Fraenkel's and was actually made public somewhat earlier.

A full confirmation of Fraenkel's work, if not of his conclusions, came from Weichselbaum.[1497-8] Beginning his studies shortly after Friedländer's first announcements, Weichselbaum covered the ground gone over by the earlier investigator, found the diplococci

in a percentage of cases sufficiently high to promise diagnostic value, and gave clear and detailed descriptions of their isolation, cultivation, and animal pathogenicity. He added the gums, tonsils, accessory sinuses, cerebrospinal fluid, and joints as sites of pneumococcal infection. The organism differed, as in Fraenkel's experience, from Friedländer's in that it failed to grow on gelatin, while the author emphatically differed from Fraenkel in the belief that this coccus was the sole cause of pneumonia.

Weichselbaum (1886)[1498-1500] separated pneumonic affections into lobar, croupous, lobular, and hypostatic pneumonias, fixed on Pneumococcus the guilt of causing the lobar type, and on streptococci, staphylococci, and a bacillus (*Bacillus pneumoniae*—probably the Friedländer bacillus) that of producing the other types. He presented new data concerning the morphology and growth characters of Pneumococcus; from the infected lung tracked the organism through the lymphatics to the cerebral ventricles; and suggested the blood stream as a further avenue of its spread. To the organism Weichselbaum gave the name *Diplococcus pneumoniae*. In the next year (1887), Weichselbaum repeated Sternberg's work and, in addition, by the subcutaneous injection of dried spleens from animals succumbing to the diplococcal infection, and of post-pneumonic pleural exudate containing attenuated pneumococci, immunized mice and rabbits. He, like Fraenkel, called the organism Pneumococcus.

In the same year, confirmation of the diversity of organisms involved in pneumonia came from Wolf,[1529] while Meyer[895] offered further proof of the presence of pneumococci in the lungs, heart, and cerebrospinal exudates in infections secondary to pneumonia. Netter,[965] finding these same cocci in the nasal fossae, sinuses, and tympanic cavity, then made the original observation that a local process, such as pneumococcal meningitis, could arise without a contributing lung infection.

Sternberg, not yet entirely convinced that the lanceolate diplococcus was the true cause of pneumonia, but hoping for a better

understanding of his work by the German bacteriologists, presented his views (1887) in a paper[1320] in the *Deutsche medizinische Wochenschrift*. He first admitted his original error in thinking that his saliva-coccus and the organism of Friedländer were the same; then went on to say that if this diplococcus could finally be proved to be the cause of true pneumonia, it should be called *Micrococcus pneumoniae crouposae*, but until that time the name he originally proposed should stand. This statement aroused Fraenkel,[471] and a month later, again feeling that he had been slighted, he took issue in print with the American, and made another plea for priority. Fraenkel characterized Sternberg's work as incomplete, made capital out of the confusion of the Sternberg coccus with Friedländer's bacterium, and disposed of Sternberg's objection to Fraenkel's name, *Pneumoniemikrococcus*.

Foà and Bordoni-Uffreduzzi[460-2] put in a bid for credit as being the first to report (1886) the discovery of the organism to which they gave the name *Diplococcus lanceolatus* in a case of primary cerebrospinal meningitis. They also reported the isolation of the organism from every case in an epidemic of the disease,* as well as from cases of polyarthritis and from the blood of the placenta in a patient aborting during pneumonia. Ortmann,[1037] too, isolated pneumococci from meningeal infections, and in so doing felt that he should be acclaimed as the first to obtain "capsule-cocci" on artificial media. Infection of the parotid glands was another manifestation of the invasiveness of Pneumococcus, two instances in which parotitis occurred being reported by Testi,[1388-90] who also noted subcutaneous abscesses as a further complication of pneumonia.

More important than the priority claim of Foà and Bordoni-Uffreduzzi, however, was their achievement in immunizing rabbits by subcutaneous injection at three or four-day intervals, first with attenuated material, then with cultures of increasing virulence. The animals thus treated became resistant to inoculation with

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* It is probable that the epidemic was one of meningococcal meningitis.

virulent cultures or infected blood. Encouraged by these results, the authors applied the procedure to human beings but without success. Incidentally, Foà and his colleague were among the first to maintain virulence of the cocci by preserving the cultures in infected blood in the cold and the dark. So also, Biondi,[117] in experiments with *Bacillus salivarius septicus* (the Fraenkel or Sternberg diplococcus in spite of the name), by chance noticed that rabbits recovering from inoculation with weakened cultures became immune. Biondi looked upon such attenuated cultures as true vaccines. Netter[971] accomplished the same result with the dried spleens of animals dying from pneumococcal infection and with "old" pleural exudate containing this organism.

Zaufal (1888)[1568-9] contributed otitis media to the list of primary infections due to Fraenkel's diplococcus, and sharply differentiated this coccus and the Friedländer bacillus, which was also found to be responsible for a similar condition. Then Gamaléia,[498] harking back to the Pasteur organism, told of its constant presence in fibrinous pneumonia, and insisted that the Friedländer bacillus was a saprophyte.

In 1889, Foà and Bordoni-Uffreduzzi[461] introduced a confusing note with the isolation of a monococcus from some mild cases of fibrinous pneumonia. They suggested the possibility of there being two types of organisms, but the descriptions are too incomplete to warrant a decision.

The names of Fraenkel and Weichselbaum had now become practically hyphenated when applied to Pneumococcus. Arustamow (1889)[24] found this organism in every one of fifty cases of pneumonia, but failed in the examination of the saliva of fifteen normal persons and the sputum from a like number of bronchitis patients. Gabbi[497] isolated in pure culture the *Virus pneumonico* (*Microbio capsulato del Fraenkel*) from a peritonsillar abscess without pneumonia; while Monti,[905] after finding Pneumococcus in an arthritic joint of the hand, induced a localized infection experimentally in a rabbit with the strain he isolated from the joint.

The decade of the eighties saw the first important chapter in the history of Pneumococcus written, edited, and given to the medical world. Its causative association with pneumonia, meningitis, and certain localized infections was accepted as established despite some confusion concerning its various identities. Fraenkel, and not Friedländer, was now looked upon as its true sponsor. The early and necessarily crude and incomplete immunization experiments were to encourage a closer study of this phase of the activity of Pneumococcus. The next ten years were to be less fruitful, but here and there facts were disclosed which, with a better understanding, were to take on a new significance.

1890–1900

In the second decade, Foà and Carbone[463] used soluble products of Pneumococcus to stimulate the immune response in rabbits, and sought by chemical means to refine and concentrate the antigenic principle—believed to be a poison or toxin—elaborated during pneumococcal growth. The authors went no further than to say that while the refined substance, which had been precipitated by ammonium sulfate and refined by dialysis, failed to kill the animal, it produced marked physiological changes—a statement which is scarcely descriptive of any specific action.

The Klemperers (1891)[728-5] might justly be looked upon as the forefathers of antipneumococcic serum therapy. They immunized rabbits with sputum obtained from pneumonia patients after recovery, with purulent, but bacteria-free, pleural exudate, with heated glycerol extracts of pneumococci, and with heated whole and filtered broth cultures. They introduced the intravenous route of injection, finding that the immunity to subsequent infection appeared far more rapidly than after subcutaneous injection—in two to three days against fourteen days. They found that duration of immunity varied from twenty-one days to more than six months. Their greater contribution was the observation that the young of immunized mother rabbits were usually passively protected, and

this observation naturally led the authors to test the curative value of the serum of actively immunized rabbits. The direct injection of such serums into the blood stream of the rabbit was effective against a lethal dose of pneumococci injected twenty-four hours later.

The Klemperers presented an array of new facts which, disregarding certain misinterpretations, have withstood the test of time and supply a rational basis for the treatment of human beings with immune serum. They observed that the serum of pneumonia patients after crisis conferred protection upon rabbits, that the protective substance came into action at the beginning of crisis, and that it neutralized the harmful properties of Pneumococcus without destroying the antigenicity of the organism. Having tested the harmlessness of immune serum on themselves, they treated six pneumonia patients with subcutaneous injections of only four to six cubic centimeters, obtaining a fall of temperature within six to twelve hours after injection, the temperature in two cases remaining normal. The Klemperers sought to explain the action of serum on an antitoxic basis, but in the light of our present knowledge it would seem doubtful if the effect was a specific one.

From bacteria-free bouillon cultures of virulent organisms, by repeated alcohol precipitation and re-solution of the precipitate in water, they isolated a protein substance, thought by them to be a pneumotoxin, which was poisonous for rabbits, withstood a temperature of 60°,* and in proper doses was capable of raising the resistance of the animals above the normal threshold of infection. In the blood of the immune animals they detected a substance not there previously, which it was conjectured must have been formed in the interval between the act of injection and the appearance of immunity, and which must be dependent upon the action of the injected albumen. The newly found substance in blood had no killing effect on living pneumococci but inhibited their toxic action. In an

* Throughout this text the figures given for temperatures represent degrees on the Centigrade scale, and the initial "C" will be omitted.

attempt to isolate this new curative substance, the Klemperers pro-
gressed far enough to be able to say that it also was a protein.
They believed that they had found an antitoxin for Pneumococcus,
but that mistake is of no importance in view of the value of their
major contribution.

The work of the Klemperers gave new impetus to investigation.
Here was an alluring and promising field; there loomed the possi-
bility of a biological cure for pneumonia. Emmerich and Fowit-
zky,[357] still using the subcutaneous route for the injection of
attenuated pneumococci, obtained only partial immunity in rabbits.
However, when they injected diluted, fully virulent cultures intra-
venously, the resistance appeared to be complete. Instead of using
the serum of these animals for protective or curative experiments,
they used the filtered juices expressed from the tissues. The fluids
so obtained were claimed to possess *idealer Heilkraft* but, although
Emmerich and Fowitzky considered their use justifiable for human
therapy, they employed the immune tissue extracts only in animal
tests. Bonome (1891)[137] tried sterile filtrates of bouillon cultures
injected every other day subcutaneously, intravenously, and intra-
peritoneally into rabbits and found that immunity appeared as
early as two or three days after the final injection. The toxicity of
the filtrates was directly related to the virulence of the strain, some
animals showing local reactions, others none. He employed, with
like effect, blood and spleen from mice killed by a culture insuffi-
ciently virulent to kill rabbits. The defibrinated blood of the
treated rabbits injected into the peritoneum of other rabbits pro-
tected them against lethal doses of the toxic filtrate, but only when
the blood was administered just prior to the injection of the fil-
trate or culture.

Kruse and Pansini[763] likewise produced immunity with daily in-
jections of sterile filtrates of broth cultures. They attributed the
protective properties of the blood of the immune rabbits to bac-
tericidins, and although they observed phagocytosis, this was held
to be a secondary factor. In their paper, apart from the immuno-

logical data, there is the account of a phenomenon, the meaning of
which the authors failed to realize, but which much later was to
assume wide biological significance. Studying forty-six strains of
diplococci of pneumonic origin under varying cultural conditions,
Kruse and Pansini noticed that the organisms when subjected to
unfavorable media, with succeeding generations began to differ
from the parent strain in morphology and colony formation. The
variations ran the gamut from typical *Diplococcus lanceolatus* to
Streptococcus pyogenes. Along with the changes in form there
took place loss of capsule and of virulence, although animal pas-
sage restored the original characters. Because of the loss of
capsule, diminution of virulence, and the appearance of chain
formation, Kruse and Pansini concluded that they had effected a
mutation of Pneumococcus into Streptococcus, and that both spe-
cies arose from a common saprophytic, streptococcal form. What-
ever may have been their conclusions, it seems certain that they
were observing the phenomenon of bacterial dissociation.

A notation of Metchnikoff's[894] was undoubtedly the first record
of the agglutination of pneumococci by immune serum. He wrote
that the microbe of pneumonia formed very long plaquettes of
"streptococci" in the serum of vaccinated rabbits.

Foà and Carbone (1891),[463] continuing their work with alcohol
and ammonium sulfate precipitates from culture filtrates, learnt
that the immunity induced by their preparations was less enduring
than that evoked by the untreated filtrates. Foà's later studies
(1893)[459] were, in a way, less productive, since, unknowingly, he
was apparently dealing with both pneumococci and meningococci.
He did show, however, that these two cocci differed from each other
in biological characters, and that the serum of animals immunized
against the one species was inactive against the other.

Here, in the matter of time and because of its bearing on the dis-
cussion in some of the preceding pages, there may be interpolated
the final description which Sternberg (1892)[1321] gave to Pneumo-
coccus under the title, *Micrococcus pneumoniae crouposae:*

ANTON W. WEICHSELBAUM 1845-1920

Discovered by the present writer in the blood of rabbits inoculated subcutaneously with his own saliva in September, 1880; by Pasteur in the blood of rabbits inoculated with the saliva of a child which died of hydrophobia in one of the hospitals of Paris in December, 1880; identified with the micrococcus in the rusty sputum of pneumonia, by comparative inoculation and culture experiments, by the writer in 1885. Proved to be the cause of croupous pneumonia in man by the researches of Talamon, Salvioli, Sternberg, Fraenkel, Weichselbaum, Netter, Gamaléia, and others.

Mosny (1892),[932] unconcerned about priority, turned his attention to immunological experiments. He grew virulent pneumococci in broth, heated the cultures at 60°, and then filtered them. The subcutaneous or intravenous injection of the filtrates into rabbits brought about immunity four days later, but the immunity was of a low order. The serum of the rabbits so treated gave protection only when injected before or at the same time with the inoculated culture. Mosny could detect no bactericidal action of the immune serum. The cocci, on the contrary, grew fully as well in immune as in normal serum. In watching the growths he noted a change in the physical appearance of the immune serum-culture mixture which we now know must have been agglutination. Mosny, like Klebs, and more particularly Metchnikoff, saw something new and told of it, unconscious that he had made a discovery.

The toxin idea was then current, and Issaeff,[673] using sterilized broth cultures of virulent pneumococci and chloroform and glycerol extracts of infected blood, interpreted the effects following their intravenous injection as being due to a toxin, but frankly confessed that the immune serum so obtained had no antitoxic properties; neither did it show any bactericidal action. The serum did, however, promote phagocytosis, and Issaeff quite rightly concluded that phagocytosis played a most important part in immunity to Pneumococcus. Emmerich[356] also entertained the toxin idea, but instead of heating or filtering broth cultures, injected them in diluted state. He stressed the desirability of using virulent strains, as well as the necessity of taking blood only from highly

immunized animals. Failure to do this would explain the unsatisfactory results of Foà and the Klemperers. Emmerich looked upon the action of the immune serum as antibacterial rather than antitoxic, and came near the truth when he explained the action as a combination of two proteins, globulin from the blood combining with a poisonous substance, probably also protein from the bacterial cell. He, like Fraenkel and Sternberg, made a bid for precedence, objecting to Foà's claim of being the originator of serum therapy.

In 1896, Washbourn,[1486] apparently unaware of Mosny's original observation, thus described the effect of adding pneumococci to specific immune serum: "When protective serum is inoculated it appears perfectly clear at the end of twenty-four hours, but at the bottom a sediment is seen. The sediment consists of pneumococci staining well and grouped in masses."

Metchnikoff's, Mosny's, and Washbourn's unnamed phenomenon was verified in 1897 by Bezançon and Griffon[108-9] and called by them "agglutination." They found that the serum of patients during pneumococcal infections acquires agglutinative power, and from their experiences they drew the conclusion, "that from the standpoint of agglutination there exist several races of pneumococci, which behave as though different microbes." Here was the basis for a method of serological classification, neglected for thirteen years until Neufeld and Haendel made it their own.

Eyre, with Washbourn (1896),[373] like Kruse and Pansini, was also close to the phenomenon of bacterial dissociation.

In old broth cultivations the majority of cocci are dead, but a few resistant forms remain living; and, by transplanting a sufficient quantity into fresh media, growth occurs and a new generation arises. We have, moreover, found that this second generation differs in morphology, biology and pathogenic properties from the parent stock. It in fact represents a distinct variety, possessing practically no virulence, and growing luxuriantly, even at 20° C., on all the usual media.

Their first attempts to bring about reversion failed, mainly

because they passed the strain through eggs. Finally, however, passage through a rabbit restored the degenerated forms to the original state of the parent strain. Without venturing an explanation, Eyre and Washbourn concluded, "Our experiments are in favor of the theory . . . that the individual cocci or their descendants actually alter in character under varying conditions," or, to use the modern term, dissociate.

The quest for a potent immune serum was continued by Denys (1897),[312] who met with greater success than did his predecessors. Denys first raised the virulence of pneumococcal cultures by serial passage of infected blood through rabbits, then administered both heated and filtered cultures to normal animals, at first rabbits, then goats, and later horses. After this preliminary treatment, Denys gave a series of injections of unheated cultures, then of the blood of infected rabbits, and finally of living, virulent pneumococci. The serum thus obtained prevented infection, was curative, and neutralized in rabbits the alleged "toxins" of Pneumococcus. The results of controlled experiments convinced Denys that the immune serum so prepared was not bactericidal, but that it contained a substance which stimulated the white corpuscles to phagocytosis, or as he phrased it, *L'immunité du lapin contre le pneumocoque, a sa source dans une modification de son sérum;* and, *L'élément immunisant primordial est le sérum et le leucocyte par lui-même n'est rien.*

Pane,[1044] in the same year, not only obtained potent serums from rabbits, cows, and asses, but tested their curative action on human beings. Of twenty-three pneumonia patients treated by intravenous injection, only two died. Pane, incidentally, noted definite agglutination in the test tube and phagocytosis in the blood and therefore was inclined to accept Metchnikoff's theory as affording an explanation for the therapeutic action of the serum.

Mennes[893] fully agreed with Denys' idea that it was the immune serum and not the normal or immune leucocyte which stimulated phagocytosis. He, also, immunized horses, gave the serum intra-

venously to pneumonia patients, and favored large doses. In the same year, a few months earlier as a matter of fact, Washbourn[1487] had injected ponies with a culture of exalted and constant virulence, and after proving to his satisfaction the favorable action of the serum on rabbits, administered it to six pneumonia patients. All, although severely ill with the disease, recovered. He made another advance by standardizing the serum, and set as a unit the smallest amount of serum which, when mixed with ten times the least fatal dose of pneumococci and injected into the peritoneum, would bring about the survival of the test rabbit. Washbourn emphasized the necessity of early treatment and advised injections of at least six hundred "units" twice daily. By himself, and in the following year with Eyre,[376] he examined immune horse serum for protective properties, and concluded that there was no parallelism between agglutinative, bactericidal, and protective power. In their papers (1899–1900),[375, 377] these two authors gave further reports on the results of potency tests made on antipneumococcic serum, including two samples of Pane's product. By this time they had modified their method, and now injected the serum intravenously and then the culture intraperitoneally into rabbits. The serums, in one cubic centimeter amounts, usually protected rabbits against a thousand or more fatal doses of pneumococci, but in a few instances failed to protect against strains from another source, owing, as we now know, to a lack of type correspondence.

Summary

At the turn of the century the results of Pneumococcus investigations could be inventoried as follows: Pneumococcus was accepted as the causative agent in lobar pneumonia; it could be grown outside the body, and some of its habits and metabolic activities were becoming known; Pneumococcus or its products were found to raise the resistance of some experimental animals to homologous infection; these animals could be made to yield a serum capable of conferring passive protection on vulnerable or stricken

creatures; and such a serum gave an early promise of mitigating pneumococcal affections in man.

The first glimpses of the vagaries of microbic behavior under varying environment were later to expand into broader views of that instability of bacterial characters which result in dissociation into varied and distinct forms or possible mutation into alien species. These discoveries were to clarify our ideas concerning virulence and the consequent infectiousness of a bacterium for a susceptible host. The fact that the serum from animals treated with Pneumococcus or some of the derivatives of Pneumococcus possesses the property of clumping this organism was, at the hands of Neufeld, Dochez, and Gillespie, and later of Cooper and her associates, to form the basis for a method of separating the members of a supposedly homogeneous species into thirty or more clearly defined and serologically specific types. This biological classification was to give a new aspect to the problems perplexing the epidemiologist, the bacteriologist and immunologist, and the clinician.

For a time Pneumococcus investigation was to lag, and then, stirred by the contributions of Neufeld and of the Cole school, it was to receive a new impetus, and to bring the vital activities of this amazing cell more clearly into sight. The facts that have been disclosed in this new and closer view, their bearing on general biological problems, and their application to the development of preventive and curative agents will be related in subsequent chapters.

CHAPTER II

BIOLOGY OF PNEUMOCOCCUS

The morphology of Pneumococcus in tissues and in cultures; isolation, cultivation, and preservation; viability and fragility, and sensitiveness to bile, soaps, and other chemical substances.

FOR the student of Pneumococcus there is no lack of reliable descriptions of its general or detailed biological features. In order, however, that the student may not be obliged to go beyond the covers of this volume for information about the organism, its intimate features will be presented here. The more important points have been freely taken from the clearest and most accurate accounts in current text and reference books, and acknowledgement is made to the respective authors.*

Morphology

The anatomy of Pneumococcus is simple and distinctive. It is best studied in preparations made from body fluids of man or animals suffering from infection. Next best are young, vigorous cultures grown on proper culture media containing blood, serum, or other body fluids. When typical, the organism consists of a pair of oval or lance-shaped cocci, their somewhat flattened proximal ends in apposition and their distal portion pointed. Sometimes single cocci are seen, while at other times single or paired cocci may be arranged in short or even long chains, resembling a string of beads —the *chapelet* originally described by Pasteur. There may be many variations from this characteristic form even when the environment is favorable. The individual cocci may be round and of

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* The sources consulted, besides original papers, are the article by Neufeld and Schnitzer in the third edition of the Kolle-Kraus-Uhlenhuth *Handbuch der Pathogenen Mikroorganismen;*[740] Zinsser and Bayne-Jones, *Textbook of Bacteriology,*[1579] *Acute Lobar Pneumonia* (Rockefeller Institute Monograph, No. 7[36]); and *A British System of Bacteriology in Relation to Medicine.*[552]

varying size (0.5 to 1.25μ), or they may be so elongated as to re-
semble bacilli. One authority describes the cells as "small," another
as "rather large," but the actual size varies with the circumstances
under which the organism is observed. In any given preparation,
along with typical forms, other members displaying every degree
of involution or degeneration may be present, while in aged cul-
tures aberrant forms may be the rule.

Pneumococcus has no spores, no vacuoles, no visible granules, no
flagella, and is non-motile. It reproduces by the primitive method
of transverse fission.

The most distinctive morphological feature of the organism is
the capsule. The capsule is more prominent when the cocci are ex-
amined in the body fluids of infected animals, or in media enriched
with body fluids, becoming faint or disappearing when the strain is
grown on less rich substrates. The capsule envelops the single,
paired, or chained cocci, frequently with a uniform periphery, al-
though sometimes it shows indentations between the twin cells or
between the paired individuals in chains.

STAINING METHODS

Pneumococcus is readily stained with the usual aniline dyes and
is Gram-positive. Exposure to the action of specific immune serum,
or even to acidulated pepsin or alkalinized trypsin mixtures fails
to rob the cocci of this property (Wilke[1520]). Only when they are
subjected to the digestive action of leucocytes, or after death and
partial disintegration in old cultures, do they give up the dye to
decolorizing agents. For peritoneal fluids, sputum, and cultures
the Gram stain is generally used. The authors prefer Burke's
modification[191] to that of Sterling* or to the original formula.[545]

Dold[324-5] recently devised a staining method by which he claimed
to be able to divide Gram-positive cocci into four groups and to
demonstrate tinctorial differences between pneumococci and strep-
tococci. He treated preparations made from cultures on solid me-

* Original reference unknown.

dia, stained by an aniline dye, phenol, and iodine, with a mixture of one part 40 per cent aqueous solution of urea and nine parts absolute alcohol. Organisms which retained the dye were called positive; those from which the dye was extracted were negative; a third class exhibited fluctuations in their ability to retain the aniline color; while the fourth group displayed periodic changes from positive to negative and vice versa. He reported that all strains of streptococci tested were without exception negative, while *Streptococcus mucosus* and thirty-nine strains of pneumococci showed a change from positive to negative or vice versa. Some of the latter gave predominantly positive results while some were usually negative with only an occasional positive reaction. These latter he considered as transition forms between the predominantly positive pneumococci and the uniformly negative strain of Streptococcus.

For demonstrating pneumococci in tissues the method described by Wadsworth* may be used to advantage, while the Gram stain is to be preferred to the Gram-Weigert technique.†[1506]

THE CAPSULE

Modern chemical study of the capsular material has given a rational basis for the serological classification of all pneumococci into definite and specific types. It is this peculiar and complex component of the pneumococcal cell that determines its specific antigenic stimulus and its immunological behavior in the presence of antibodies. This knowledge enables us to utilize in a far more intelligent way Pneumococcus or its components in the induction of active immunity and consequently in the production of immune serums, and furnishes us with a delicate reagent for measuring the body's response to various immunizing procedures, and for determining the potency of antipneumococcic serums.

The capsule interested and perplexed many of the earlier investigators. Described first by Pasteur[1066] as an *auréole*, and by Fried-

* Zinsser and Bayne-Jones.[1579]
† Neufeld prefers the Gram technique; Zinsser the Gram-Weigert technique.

länder[486-7] as a *Kapsel*, it was later called a rim, or a hull, and its presence undoubtedly contributed to the confusion between the cocci first described by Friedländer and the well-known bacillus that bears his name. Because of the inconstancy of its appearance particularly under different conditions of artificial cultivation, the belief arose that it was not a diagnostic feature of the species. Pane[1045] went so far as to assert that it was a degenerative and not a developmental phenomenon. Friedländer had always contended, however, that the capsule was the product of a vital function of the cell.

Aoki[16] noticed that capsule formation proceeded more rapidly at body than at lower temperatures, and that the capsules were larger and more distinct when the cocci were grown in immune serum or in media containing such serum. This was the *Quellung* effect utilized by Neufeld and Etinger-Tulczynska[988] in their recently published method for type determination. The capsule was often described as gelatinous but, since the time of Pasteur and Friedländer, it had been more generally believed to consist of mucin. It was Preisz[1108] who, acting upon this assumption, was the first to demonstrate capsular material in the blood and other body fluids of animals succumbing to pneumococcal infection. He believed that a general, fatal invasion of the body by these cocci could occur only when the accumulation of mucin—now soluble specific substance—in the body had reached a certain level.

STAINING THE CAPSULE

Many methods have been devised for demonstrating the capsule of Pneumococcus. The first record of a capsule stain for Pneumococcus is that by Friedländer (1885)[491] who, after fixing the preparation in the flame, dipped it for a few minutes in one per cent acetic acid. After blowing off the acid he dried and then stained the film by a few seconds' exposure to a saturated aniline-water gentian violet solution. MacConkey (1898)[843] recommended staining with dahlia, methyl green, and a saturated alcoholic solution of

fuchsin. The method, he claimed, gave a clear image suitable for photography. Hiss[650] in 1902 gave two procedures, the first, a gentian violet-potassium carbonate method, the other, and a better one, a preliminary treatment with gentian violet or fuchsin followed by washing with 20 per cent copper sulfate solution. The Welch* formula of glacial acetic acid, followed by aniline-water gentian violet gives clear pictures of the capsule. These methods are widely used and give satisfactory results.

Buerger (1905)[168] described a different and also reliable procedure, but the intricacy of the technique prevented general adoption. Malone,[860] after a preliminary staining with Congo red, treated the film with dilute hydrochloric acid in alcohol, counterstaining with methyl violet. Malone claimed that by this technique he was able to demonstrate capsules when the Hiss method failed. Wherry[1515] mixed a suspension of pneumococci in diluted serum with an equal amount of 0.05 normal hydrochloric acid, spread the mixture in a thin film, fixed the film with heat, and stained it quickly with carbol gentian violet. Leifson[797] applied to films a mixture of a saturated aqueous solution of ammonia or potassium alum, tannic acid in water, 95 per cent ethyl alcohol, and saturated alcoholic solution of basic fuchsin. He followed this treatment with methylene blue in aqueous borax solution. This method never came into general use. The Huntoon* technique is suitable only for cultures and not for animal exudates. India ink is the choice of some bacteriologists. It is a negative stain, in that the medium on the slide is colored black, while the cells and the capsule are unstained. Lévy-Bruhl and Borin,[805] however, reported that this stain outlined the capsules on Type III organisms when no albuminous substances were present in their "T" medium, but failed with other types of pneumococci grown on this special substrate. For general purposes, however, the Burke or Sterling modifications of the Gram stain suffice, while the Gram stain, the Gram-Weigert, or the

* Zinsser and Bayne-Jones.[1579]

method described by Wadsworth* may be used to demonstrate capsules in tissue sections.

The capsules are indicative of the vigor and virulence of the strain. They may best be seen "in the blood, serum and inflammatory exudate of the infected rabbit and white mouse. They are equally well marked in the fresh sputum of pneumonia patients, especially in the early stages of the disease, and in the exudate accompanying such pneumococcus infections as meningitis, otitis media and empyema."*

The addition of blood, serum, milk, or other body fluids to nutrient media favors the development of the capsule under artificial cultivation.

Isolation of Pneumococcus

ANIMAL INOCULATION

One of the simplest ways of isolating pneumococci from infected material is by mouse inoculation. If the source material is sputum, a small portion can be washed in sterile broth or saline solution in a Petri dish to remove the majority of accompanying mouth organisms. The washed sputum together with a small quantity of broth is emulsified by grinding in a sterile mortar and the suspension in amounts of 0.5 to one cubic centimeter injected into the peritoneal cavity of a white mouse. Just before or shortly after death of the animal, the heart is exposed and, under sterile precautions, punctured with a capillary pipette or hypodermic needle, and the blood aspirated and planted in broth and on blood-agar plates. In this way a pure culture can usually be obtained at the first trial. Pieces of lung or other infected material may be treated in the same manner as sputum. When it is suspected that few pneumococci are present, the tissue may be macerated in broth and given a preliminary incubation before the mouse injection.†

* Zinsser and Bayne-Jones.[1579]
† For complete details of the method see Appendix.

Instead of the practice of animal inoculation, washed sputum, blood, or broth suspensions of infected material may be streaked on plates poured with fresh blood agar or serum-dextrose agar. For the cultivation of organisms directly from the circulating blood, the blood withdrawn by venous puncture may be added to a fairly large volume of dextrose broth in flasks, or mixed with molten dextrose agar and poured into Petri dishes. Pneumococcus, being facultative in its oxygen needs, grows on the surface as well as in the depths of the medium.

From agar plates single colonies of pneumococci may be picked and replanted on agar, seeded on serum or blood-agar slants or in serum or glucose broth. These media serve for the further propagation of the original broth cultures when pure. Small inoculums grow more successfully on solid than on liquid media. Incubation should be carried on at a temperature of about $37.5°$; $25°$ and $41°$ represent the lower and upper limits favorable to growth. A rarity were the strains isolated from blood and exudates by Eaton* in Zinsser's laboratory, which grew only at $25°$. At $37.5°$ the organisms failed to grow unless incubated in an atmosphere containing about 10 per cent of carbon dioxide. A somewhat similar strain was that previously reported by Kindborg.[712] Isolated from rusty sputum by mouse inoculation, the organism displayed all the characters of a typical pneumococcus, yet it grew readily at $22°$ and rapidly and energetically liquefied gelatin. While pathogenic for mice, it was avirulent for rabbits.

Cultivation

Pneumococcus, being a strict parasite, is somewhat fastidious in nutritional requirements, as well as sensitive to the physical and chemical conditions of its surroundings. The essential ingredients of culture media are meat extractives prepared from fresh muscle

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* Quoted by Zinsser and Bayne-Jones.[1579]

tissue, protein of comparatively small molecular size in the form of peptone, a small amount of sugar, mineral salts, and a suitable concentration of hydrogen ions. All media should be subjected to the least degree of heat necessary to effect extraction of the meat, to render the ingredients soluble, and to ensure final sterility.

The earlier bacteriologists employed the only media known in their time, such as egg albumen (Klebs[718]), veal or beef broth (Pasteur), then broth with the addition of gelatin or agar (Friedländer[487]) and later of blood (Nissen,[1013] and Gilbert and Fournier,[513] among others), serum, or other body fluids. Fraenkel[469] employed milk, but finding the cocci lost virulence on this medium, turned to coagulated blood serum and then to meat-infusion agar. Eggs, both in the shell (Bunzl-Federn[186]), inspissated, and as a base with broth (Gaskell[499]) have been employed for the maintenance of virulence, while Grawitz and Steffen[547] preferred coagulated sputum as a favorable medium for capsule formation. Schmidt[1236] also recommended sputum as a medium on which Pneumococcus developed clear, well-formed capsules, and which also would restore capsules to cocci that had lost them after cultivation on agar. Gilbert and Fournier[513] found defibrinated blood of man and other animals to be suitable for the propagation of pneumococci. Carnot and Fournier[200] noted constancy of virulence and capsule formation of strains grown in broth and on agar containing brain tissue, but the fluid medium could be employed only for the carrying on of pure cultures.

Among the great variety of nutrient materials recommended, time and experience have shown the essential ones, and these are now incorporated into a few formulas which meet all the needs of the student of Pneumococcus.* While Pneumococcus can be grown within a fairly wide range of cultural conditions, the most vigorous and viable individuals are obtained when careful attention is given to the needs peculiar to this organism. Fresh beef, veal, or

* See Appendix.

horse muscle, freed from fat, furnish a better base than the com-
mercial meat extracts. Peptone preparations containing the higher
proportion of proteose nitrogen, mainly because of the stronger
buffering action, are to be preferred to preparations in which the
bulk of the nitrogen is in the form of peptones and amino acids.
The latter, however, because of their lower cost may be substi-
tuted for routine purposes. In different formulas the amount of
peptone varies from 0.1 to 2 per cent, depending upon the pro-
teose nitrogen content of the preparation. Felton and Dougherty[425]
claimed that 2 per cent of peptone preserves and increases viru-
lence.

Kruse and Pansini[763] first drew attention to the fact that certain
preparations of peptone hindered full growth, a problem later
studied by Wright[1549] in 1933, who pointed out the fact that one
of the difficulties in preparing suitable broth was largely due to in-
complete reduction of peptone. This obstacle could be overcome by
adding the peptone to the broth before heat is applied, thus ex-
posing it to the reducing action of meat or meat infusion during
the steaming process. The inhibitory action of peptone may be fur-
ther diminished by the reducing action of sugars and an alkaline
reaction (Wright). Dubos[333-4] also found that some commercial
peptones contain bacteriostatic substances. These may be removed
by precipitation with acid and acetone, or their inhibitory action
may be neutralized by the addition of reduced thiol compounds
(0.0003 per cent thioglycollic acid). Reduced cysteine increases
the rate of growth.

A small amount of sodium chloride, usually 0.5 per cent, ap-
pears to be a necessary ingredient, although sodium phosphate
may be substituted. An excess of salt retards or prevents the
growth of Pneumococcus (Wright[1548]), and its concentration,
whether as chloride or phosphate, should not exceed 0.1 molar
(Dernby and Avery[313]). Glucose stimulates and enhances initial
growth, and quantities ranging from 0.1 to 4.0 per cent have been
advised, while Turro[1430] preferred 8 per cent! Amounts higher than

one per cent, because of the glycolytic action of Pneumococcus with the resulting acid production, may produce retardation or complete inhibition of growth and bring about autolysis (Wright[1548]). The presence of muscle sugar in the meat base must be reckoned as an additional sugar factor when fermentation reactions are to be quantitatively measured. McGuire, Valentine, Whitney, and Falk[879] advised titration of the fermentable reducing sugar in muscle (beef heart) infusion and, taking the equivalent of 0.5 milligram of glucose per cubic centimeter as the lower limit, then added glucose to the desired concentration. Glycerol may replace glucose or other sugars as a source of carbohydrate material, but glycerol, like the other sugars, is susceptible to the fermentative action of Pneumococcus and yields acid on enzymatic cleavage.

The acid formed in broth by the action·of Pneumococcus may be neutralized either by the addition of sterile, powdered calcium carbonate (Wurz and Mosny,[1554] and Hiss[650]), or of small pieces of marble, washed and placed in the test tubes before filling and sterilization (Bolduan[135]).

The initial hydrogen ion concentration of the medium and the changes occurring during growth are vital factors in the proper cultivation of the organism. According to Dernby and Avery,[313] culture media should have an initial reaction represented by a pH of 7.8 to 8.0 with an optimal pH of 7.8. They gave the limiting hydrogen ion concentration for initiation of growth for the various types of Pneumococcus as pH 7.0 to 8.3. Strains will live but will not actively multiply in substrates having a degree of acidity or alkalinity greater than those represented by these figures. Lord and Nye[829] have given further information regarding the relation of hydrogen ion concentration to the life, growth, and death of Pneumococcus. As the medium becomes more acid (pH 7.4 to 6.8) the organism may live many days; between 6.8 and 5.1 death begins to take place—the greater the acidity, the more rapid the death, while at 5.1 the cocci die within a few hours. In cloudy sus-

pensions of washed pneumococci dissolution is most marked within the range pH 5.0 to 6.0. Some disintegration occurs toward the alkaline side but none at the most acidic end of the scale.

For the successful initiation of growth in broth, once a pure culture is obtained, a fairly heavy amount of inoculum is advisable. Cole used 0.1 cubic centimeter for every cubic centimeter of broth. These amounts are obviously impossible when cultures are to be planted from single colonies or from mouse-hearts' blood and similar materials. When original infectious material is used as inoculum a small amount usually suffices. A generous planting, however, favors multiplication.

The cycle of pneumococcal growth in broth has been studied by Chesney.[220] When broth is inoculated with a culture that has passed the stage of maximal growth, there ensues a latent period before growth appears in the freshly seeded broth. When, however, inoculation is performed with cultures at their maximal rate of growth, there is no delay in multiplication. This initial latent period or lag—that is, the interval between the time of seeding and the time at which maximal rate of growth begins—is followed by a phase of rapid growth, during which the organisms are dividing regularly. This stage is followed by a stationary period, in which the organisms cease to multiply at maximal rate so that the increase in number becomes slower and finally ceases and, although they remain viable, the number of cells present in a unit volume remains approximately constant for an appreciable length of time. Finally the period of decline sets in, in which the number of living organisms begins to decrease. Chesney believed that this lag is an expression of injury which the bacterial cell sustains from previous environment.

The failure of pneumococci to continue to grow and multiply after the stationary period is due to the production of acid or possibly of other inhibiting substances. Morgan and Avery[915] listed the inhibiting factors as the accumulation of acid products, the exhaustion of nutritive substances, and the formation and accumu-

lation, under certain cultural conditions, of peroxide in the medium. Hager,* by daily neutralizing the newly formed acid has succeeded in prolonging the period of growth and, therefore, in obtaining unusually heavy yields of pneumococci from a single lot of broth.

Felton and Dougherty[423-4] contrived an ingenious apparatus for delivering a continuous supply of pneumococci at the stage of most active growth. By an automatic device, an inoculum of vigorous, young culture was removed from the exhausted medium and planted in a flask containing a fresh food supply of milk. The transfers were made at two, four, and eight-hour intervals. Propagation by this method at eight-hour intervals raised the virulence of the strain to a degree unprecedented in any former *in vitro* experiments. Methods for obtaining large amounts of living pneumococci for chemical study as well as for other purposes have been described by Felton and Huntoon,[667] while that of Hager has not yet come to publication.†

These media, varying in their constituents, all require heavy inoculation with a vigorous culture (Gillespie[515]), careful initial adjustment or subsequent correction of the reaction and, of course, incubation at a constant temperature of about 37.5° if they are to give a large yield. Dubos[331] explained the necessity for large amounts of inoculum in seeding broth as being based on the establishment of a proper reduction potential in the medium. This condition is brought about by the large inoculum owing to the reducing properties of the bacterial cells.

ACCESSORY SUBSTANCES

Optimal growth conditions, that is, as far as rate of growth, number, and viability of the organisms are concerned, can be provided beyond those already mentioned by the addition of a variety of substances. Autoclaved gelatin, by its buffering action in addition to its nutritive elements, has been found by Platt[1096] to induce

* Personal communication.   † See Appendix.

and sustain growth of Pneumococcus in otherwise unsuitable media. Robertson, Sia, and Wood[1149] ascertained that the protective action of gelatin lies largely in shielding pneumococci against physical injury—possibly the toxic action of electrolytes—which occurs during dilution in solutions of crystalloids. Furthermore, gelatin exerts a well-marked preservative action of unknown nature in protecting the organisms against early dissolution.

One of the most commonly used substances is blood, of either man, rabbit, horse, or sheep. Pasteur, Sternberg, and Levy and Steinmetz[802] early recognized its value. Citrated, laked, or, better, defibrinated blood augments the growth-promoting value of the substrate. It is best used in a ratio of one part or less to ten to twenty parts of medium. Normal serum, transudates, and ascitic and hydrocele fluids similarly supply elements favorable to the growth of Pneumococcus, and are common ingredients in many media formulas (Fraenkel,[468] Behring and Nissen,[98] Mosny,[933] Levinthal,[800] and many others).

In a study of the growth-promoting properties of serum from a variety of animal species, Bezançon and Griffon[112] found that, in general, growth was more rapid and abundant in the serum of animals susceptible to pneumococcal infection. However, in these serums cell death took place rapidly after maximal growth had been reached. In the serum of resistant animals, on the other hand, growth was meager, although the organisms remained viable and virulent for a longer period of time. The serum of young animals, whether of a sensitive or refractory species, possessed the growth-promoting properties of susceptible species, whereas the serum of older animals was less favorable to growth.

Bordet[139] has recently described an unexplained and as yet unconfirmed cultural and morphological appearance of cultures in rabbit serum. The growth of pneumococci had a milky appearance, and at the bottom of the tube a sediment formed which consisted of a clustered mass of large round elements. These bodies failed to take well the basic stains but were readily stained by neu-

tral dyes. They were variable in size and had fairly regular out-
lines and a granular structure. Inasmuch as these bodies did not
appear in serum media previously heated at 56° for one-half hour,
but did appear when the temperature was no higher than 50°,
Bordet thought that their production was due to alexin, although
they were not present in broth containing serum from rabbits vac-
cinated against Pneumococcus, either of homologous or heterolo-
gous type.

Blood and serum, in addition to contributing some nutritive
substances, act as buffers in controlling the reaction and, being
colloids, arrest any toxic action of inorganic salts. Blood, because
of its oxidation-reduction system, tends to maintain a proper oxy-
gen balance, while the iron in the hemoglobin seems to act as a
catalase. In studying the supposed necessity for hemoglobin for
the growth of *Bacillus influenzae*, Thjötta[1391] discovered that this
substance could be omitted if extracts of mucoid bacilli or of *Ba-
cillus proteus* were substituted. Thjötta looked upon the accessory
substance in such extracts as a vitamin which, in many ways, cor-
responded to Wildier's "Bios" of yeast, the "auxomones" of Bot-
tomly, or the hormones. Then Thjötta and Avery[1392-3] learnt that
these substances—the "V" factor—alone would not support the
growth of hemophilic bacteria through several generations, but
that another substance, the so-called "X" factor, contained in red
corpuscles was essential. This X factor is heat-stable and acts in
minute amounts. Both the V and X factors are required for the
continued and complete development of Pneumococcus as well as
for the growth of hemophilic organisms.

Baudisch and Dubos[89] investigated the influence of iron com-
pounds on the viability of pneumococci. Small amounts of iron
oxide prolong the life of avirulent strains but are less favorable to
virulent strains. Iron may be stimulating or harmful depending
upon the particular chemical radical with which it is combined.

Another accessory substance of animal origin, recommended by
Quiroga,[1115] is liver. The addition of an extract of beef liver to

peptone broth or Martin bouillon gives early growth with less pro-
duction of acid than that which occurs when glucose is used. In
such a medium it is probably the glycogen or other sugars which
supply the stimulus, while the tissue itself and the hemoglobin fur-
nish the other growth-promoting factors. The protective action of
muscle tissue is exemplified in the hormone-blood agar and hor-
mone-gelatin broth of Bailey.[67] The formula is a modification of
that of Huntoon[664] for the preparation of original hormone me-
dium, and both methods, and also that of Douglas for making
tryptic digest medium, yield substrates well suited not only for the
prolific growth of pneumococci but for conservation of essential
biological characters of the organism.

Still another factor to be considered is the oxygen requirement
of Pneumococcus. The organism is a facultative anaerobe, and
grows both in the presence and absence of oxygen. Examples of
anaerobic strains of pneumococci have been encountered by Avery
and his associates,* while two similar strains which apparently
constitute two new serological types have recently been described
by Smith.[1296] Because of its action in elaborating toxic peroxides
in the surrounding medium, as first demonstrated by McLeod and
Glovenlock[885] and later proved by McLeod and Gordon[881-4] to be
hydrogen peroxide, Pneumococcus may hinder or entirely prevent
its own growth. The inhibiting effect of the peroxide may be over-
come by the addition of plant tissues or other substances exerting
an oxidizing-reducing action (Avery and Morgan[52]).

VEGETABLE ACCESSORY SUBSTANCES

Avery and Morgan[51, 53] found that the addition to broth of ster-
ile, unheated plant tissue, such as yellow and white turnip, carrot,
beet, parsnip, white and sweet potato, and banana,† not only
caused acceleration of pneumococcal growth, but served to induce
abundant multiplication even when the seeding was too minute to

* Personal communication.
† Falk, Valentine, McGuire, and Whitney[388] have more recently (1932) rec-
ommended the use of the green banana as enriching material in the place of

initiate growth in the medium. With potato broth the beginning period of growth was prolonged and cell death delayed. Moreover, in plant-tissue medium, the zone of hydrogen ion concentration within which growth could be initiated was considerably extended beyond the optimal range in ordinary bouillon. Fresh plant tissue, that is tissue which has not been subjected to heat or oxidation, was necessary for this effect, and this fact explains the action of the tissues in promoting the growth of certain anaerobic bacteria in the presence of oxygen. In the case of Pneumococcus, the action of the oxidizing-reducing system of the V and the X factors* leads to the destruction of toxic peroxides.

Yeast, in the form of autolysate, in combination with clotted horse blood, peptone, agar, glucose, and maltose was found by Hitchens[654] to be a favorable medium for promoting the growth of pneumococci.

APPEARANCE OF GROWTH

In a suitable broth, the first evidence of growth is a faint, uniform clouding of the medium. There is a slow deposition of a light, flocculent sediment at the bottom of the container. On further incubation there is a gradual clearing of the medium with the accumulation of a heavier deposit, while some of the falling particles may adhere to the side of the vessel. There is no pellicle formation. Growth is more rapid and luxuriant when glucose, the accessory substances already mentioned, or some sterile body fluid are added.

In milk, Pneumococcus thrives and develops capsules, and if transfers are made at six or eight-hour intervals, the vigor and virulence of the organism are maintained. Prolonged cultivation in this medium without frequent transplants, however, is detrimental to the cocci owing to the acid produced. Milk is coagulated by

fresh blood in beef-heart broth for the cultivation of pneumococci, while Larson and Thompson[786] employ broth containing sterile, fresh, unheated potato extract for growing pneumococci for type determination and bile-solubility tests. The latter medium has the advantage over serum or blood broth of being free from extraneous substances which might interfere in these tests.

* See p. 43.

practically all strains. Pneumococci will grow in gelatin at 37°
but, because gelatin melts, it is not a useful medium for propagat-
ing pneumococci.

On agar, Pneumococcus grows in very small, round, water-clear,
non-confluent colonies, resembling in many respects those of strep-
tococci, but differing from them in being more transparent, more
moist, and flatter. Under the microscope, colonies of Pneumococ-
cus are seen to be finely granular, with dark centers fading to
lighter zones nearer the periphery, which is regular, sometimes
slightly wavy, but never showing the intertwined convolutions such
as those seen in colonies of Streptococcus. In agar stab cultures
and sub-surface agar plate cultures, the colonies appear within
twenty-four to thirty-six hours, are smaller, sometimes almost in-
visible, and indistinguishable from colonies of Streptococcus. Un-
der low magnification they show partly as yellowish-brown, partly
as bright, oval, lenticular or whetstone-like forms.

On blood-agar plates the colony may be surrounded by a zone of
methemoglobin formation with an exterior zone of greenish color
after twenty-four or more hours of incubation. Later a zone of
hemolysis may appear at the margin fainter than that encircling
streptococcal colonies, and this appearance may lead to error in
the differentiation of the two organisms. When grown on "mix-
tures of whole rabbits' blood and agar, the pneumococcus grows
well, and forms, after four or five days, thick, black surface colo-
nies, not unlike sun blisters on red paint. These colonies are easily
distinguished from those of streptococci, and are of considerable
differential value (Hiss)."*

"Upon potato, thin, moist growth appears, scarcely visible and
indistinguishable from an increased moisture on the surface of the
medium," making this an unsuitable substrate. "Upon Loeffler's
coagulated blood serum, the pneumococcus develops into moist,
watery, discrete colonies which tend to disappear by a drying out

* Paraphrase and direct quotation from Zinsser and Bayne-Jones.[1579]

of colonies after some days, differing in this from streptococcus colonies."*

DIFFERENTIAL MEDIA

In addition to the diagnostic features of pneumococcal colonies grown on Loeffler's medium and on the rabbit blood-agar mixture, other media have been recommended as serving to develop characters that distinguish pneumococci from streptococci. Hiss,[650] for this purpose, devised a medium consisting of one part of beef serum and two parts of distilled water, to which was added one per cent of inulin (C.P.) and enough litmus to render the medium a clear, transparent blue. By fermentation of the inulin, Pneumococcus acidifies the mixture, causing coagulation of the serum. The method is useful except in the case of those rare strains of pneumococci which are not inulin fermenters. A similar, but solid, medium for the same purpose was that recommended by Ruediger.[1195] To sugar-free broth, containing one per cent of Witte peptone and 1.5 per cent of agar, he added approximately 1.5 per cent of pure inulin and a small amount of Merck's highest purity litmus. One cubic centimeter of ascites fluid heated to $65°$ was added to each tube of melted agar. Pneumococci are distinguished by the formation of red colonies on this medium.

Buerger[163] claimed that the ring type of colony on serum, or better serum-glucose agar, was distinctive of Pneumococcus when compared with colonies of Streptococcus on the same medium. The method required a close inspection of the growth under transmitted and reflected artificial light. When viewed from the side or by transmitted light, the pneumococcal colony shows a distinct milky ring enclosing a transparent center, while the streptococcal colony has a prominent periphery and a definite nucleus. These and other physical characters serve to differentiate these two species of cocci.

* From Zinsser and Bayne-Jones.[1579]

Bieling,[114] by the use of laked blood agar, laked blood-optochin agar, and boiled blood agar, could differentiate pneumococci, and the *longus* and *mitior* types of streptococci. Presting[1109] used the Bieling method in studying some sixty carefully identified strains of pneumococci and streptococci, and concluded that boiled blood agar was a suitable aid in differentiating pneumococci and green-growing and hemolytic streptococci. On the laked blood agar the hemolytic streptococci exhibited such marked variation that *viridans* could not be distinguished from the hemolytic types, nor from pneumococci. Koch,[731] after one year's favorable experience with Bieling's blood-optochin agar, recommended the medium for the differentiation of pneumococci and streptococci.

The "polytrope" medium of Lange—a special lactose-mannite-peptone bouillon—as reported by Haendel and Lange,[585] develops a diffuse orange-yellow coloration when pneumococci of long artificial cultivation are grown on it, but remains uncolored when the strains are cultivated directly from infected animal material. This latter fact renders this medium unsuited to the differentiation of bacterial species, because streptococcal strains of long cultivation, and other bacterial species as well, cause no color change. Dog-blood agar plates, according to Sia and Chung,[1270] give colony differences sufficiently pronounced to enable one to distinguish between colonies of smooth and rough strains of pneumococci. For the same purpose, the authors, in a later communication,[1271] reported that a medium consisting of one per cent dextrose-beef-infusion agar with a reaction equal to pH 7.8 containing type-specific antipneumococcic serum served to identify the pneumococcal type strain. In poured plates, the colonies under the surface, when illuminated from the side on a dark background, showed an annular opacity which was claimed to be type-specific.

Thomson and Thomson,[1400] by means of photomicrographs of colonies of pneumococci grown on plasma-testicular agar, claimed that it was possible to detect significant species and even type differences. Their excellent photographs repay study.

Viability

The factors already described, along with temperature conditions, determine not only the speed and mass of growth but the span of life of Pneumococcus. In highly buffered fluids such as blood, blood serum, or media containing these body tissues, Pneumococcus can be preserved alive and virulent for long periods of time. Arkharow (1892)[17] found that blood from infected mice and rabbits retained its virulence if kept in sealed tubes in the dark at room temperature. Foà and Bordoni-Uffreduzzi (1888)[462] incubated the blood of an infected rabbit for twenty-four hours and then stored it in the dark in the cold. Vitality was preserved for fifty to sixty days. Rymowitsch[1199] accidentally noted that hemoglobin in the medium would conserve viability and virulence for an equal length of time at 36° to 38°. Yourevitch[1566] proposed a method, based on the protective action of blood and tissue, for preserving pneumococci in latent culture. Blood is aspirated by a pipette and expelled into the bottom of tubes of glucose or serum broth, or a whole heart or fragments of clotted rabbit blood may be added. After planting, the cultures are hermetically sealed and kept in the ice-box. In such cultures both viability and virulence remain unimpaired for several months.

Römer[1155] preserved pneumococci by mixing the heart-blood of a rabbit dead from pneumococcal infection with sterile saline solution and storing the infectious material in sealed tubes in a cool, dark place. He maintained the virulence of the stock material by weekly passage through rabbits.

Washbourn,[1486] dissatisfied with these methods, preserved stock cultures as long as fifty days by covering the growth on agar cultures with a thin layer of rabbit blood. Gilbert and Fournier[513] kept strains viable for two months by simple inoculation in fluid, defibrinated blood. The cocci still showed capsules and were virulent for mice. Bezançon and Griffon[112] modified Gilbert and Fournier's procedure by injecting a proteose solution in dogs, then mixing their blood with ascitic or pleuritic fluids before seeding

with pneumococci. Incubated continuously at 37.5°, the pneumo-
cocci survived for four months. Truche and Cotoni[1422] added two
volumes of 15 per cent gelatin in physiological salt solution to
cultures grown at 37.5° in the "T" medium, sealed the tubes, and
kept them in the ice-box. In this way viable stock cultures could be
maintained for at least six months. Ungermann[1435] cultivated pneu-
mococci in concentrated rabbit serum and then protected the cul-
tures with a layer of sterile paraffin oil, with incubation at 37.5°.
Later, Truche[1421] modified Ungermann's method by first growing
strains on blood or serum agar, and then covering the surface
with formalinized serum. Preservation continued for a year, yield-
ing virulent material for inoculation. In a semi-fluid medium con-
sisting of one part of nutrient agar and five parts of sterile ascitic
or pleuritic fluids, with short incubation and storage at 8° to 10°,
Wadsworth (1903)[1454] preserved cultures in a viable condition for
three or more months. In these exudates without agar, the period
of viability was even greater. A similar medium with the addition
of glucose was described in 1930 by Vélicanoff and Mikhailova.[1450]

Dehydration is an excellent physical method for the preserva-
tion of cultures of Pneumococcus, particularly in those cases in
which experiments continued over a long period of time require
that the characters of the strain be held uniform and constant.
Nissen (1891)[1013] first applied this principle to the drying of ster-
ile silk threads saturated with broth and serum-broth cultures, but
the results were only indicative of the success which was to attend
Heim's[631] efforts fourteen years later. Similar threads, soaked in
the blood of a cat dying of pneumococcal septicemia and dried
over calcium chloride in a desiccator, fourteen and sixteen months
later yielded in broth a growth of pneumococci possessing a viru-
lence equal to that of the original material.

In the year of Nissen's publication, Bordoni-Uffreduzzi,[142] not
seeking a method of preservation, reported that pneumonic sputum
when dried on linen rags gave growth after twelve days' exposure
to direct sunlight, and as late as after fifty-five days of exposure

to diffuse daylight. They concluded that the blood and proteins in the sputum formed a protective coating for the cocci. Wood[1542] studied the longevity of Pneumococcus in sputum when subjected to varying temperatures. In moist sputum, kept in the dark at room temperature, the average life of Pneumococcus is eleven days, while at $0°$ it is, as a rule, thirty-five days. When the sputum is kept in strong light at room temperature the life span is less than five days. In dried sputum Pneumococcus lives, on an average, thirty-five days in the dark, thirty days in diffuse daylight, and less than four hours in sunlight. In powdered sputum, death is a matter of a few hours. Pneumococci in sprayed, moist sputum rarely survive for more than an hour, and often die in less time. The material upon which the sprayed particles fall has little influence on the life of the organism. On the contrary, Emmerich[355] and more recently Stillman,[1326] have demonstrated the longevity and continued virulence of pneumococci in a dried condition. Stillman* recovered viable organisms from pneumonic sputum dried in test tubes and exposed for three months or more to diffuse daylight at room temperature. Germano,[511] too, found pneumococci resistant to drying, but the details of his experiments are lacking. Hintze[646] kept blood-agar cultures alive for thirty-two months by storage in tubes stoppered with cotton plugs. For the purpose of keeping pneumococci viable and virulent, Savino and Acuna[1220] placed the blood of mice or rabbits infected with Pneumococcus in one arm of an H tube with phosphoric anhydride in the other arm and then sealed the tube after evacuating the air.

The thermal death-point for Pneumococcus as given by Sternberg[1321] is $52°$ for ten minutes.

For practical purposes, however, such as the maintenance of virulent stock cultures or of other infectious pneumococcal material, in addition to refrigeration of inoculated blood in sealed tubes and the devices already described, the rapid drying of cultures, infected organs, blood, or sputum presents many advantages.

* Personal communication.

Swift's[1370] method is a successful example. The culture is scraped from the surface of the solid medium, spread in thin layers on plates or in tubes, and quickly frozen. The frozen material is then placed in a chilled Hempel desiccator containing phosphorus pentoxide in the bottom and sulfuric ether in the moat. The drawing of a high vacuum dissipates the frozen moisture, and the resulting dry powder, if kept anhydrous, is found to contain viable organisms after many months of storage. Later, Otten,[1040] using a similar method for drying thick suspensions of pneumococci in salt solution, reported their recovery in living condition after long periods of preservation. Desiccated cultures, unlike those on moist media, can be shipped over distances involving many days' exposure to wide temperature variations.

The reader who desires further details concerning the preservation of bacterial cultures is referred to the communication of Flosdorf and Mudd[454] who, after reviewing the literature on the subject, described an apparatus for the rapid drying and preservation of bacteria, serum, and other biological substances. The method consists in applying a high vacuum to the material to be dried rapidly and freezing it by immersion in a bath chilled to $-78°$ by the use of Dry-Ice and Methyl Cellusolve.

Another practical method for having virulent cultures at hand is that devised by Neufeld[1000] based on the principle earlier established by Heim. Pieces of spleen or heart or the whole organs of mice dead of experimental pneumococcal infection are placed in open Petri dishes or in small tubes loosely plugged with cotton and dried in a vacuum desiccator over calcium chloride or concentrated sulfuric acid. To recover the pneumococci, generous pieces of the dried organs are ground in a mortar with broth, and the suspension injected intraperitoneally into a mouse. The heart's blood of the dead or dying mouse usually yields a pure, virulent culture.

Autolysis

Pneumococcus is a delicate organism and may rapidly disinte-

grate under certain physical conditions of the medium which favor the action of its own lytic ferments. Bürgers[187] was among the first to study the self-dissolution of pneumococci. He reported that autolysis, while favored by the addition of chloroform, was prevented by heating the organisms to 60° or above. One of the environmental conditions which influence dissolution is the degree of acidity of the substrate. Mair[552] gave the range of hydrogen ion concentration within which autolysis occurs as from pH 8.5 to 6.0, with an optimum of pH 6.8. Lord and Nye[829] observed the dissolution of suspended living pneumococci of Types I and II in isotonic standard solutions and in approximately isotonic bouillon having reactions between pH 4.0 and pH 8.0. Lysis took place with a reaction higher than pH 5.0 and was most marked in the range pH 5.0 to 7.0. It was still observable on the alkaline side, but was absent when the acidity was greater than pH 5.0. This phenomenon takes place at ice-box, room, and incubator temperatures. Mair found the rate of lysis increased to a maximum at 42°, and stated that this maximum was due to partial destruction of the autolytic ferment at higher temperatures, and also that the autolysin was more sensitive to heat when the reaction was alkaline. Exposure of pneumococci to 47° for one hour diminishes the degree of dissolution, while an exposure of thirty minutes at 56° or five minutes at 100° completely arrests the action. Inasmuch as Sternberg[1321] determined the thermal death-point as 52° for ten minutes, if the observation was correct, the lower temperature should suffice to prevent self-lysis, but such is not the case.

The addition of fresh human serum to suspensions of the cocci at varying reactions prevents autolysis. Solutions of lysed pneumococci added to fresh suspensions of pneumococci in standard solutions of the same hydrogen ion concentration increase autolysis. Cultures of *S. viridans*, *S. haemolyticus*, and *Staphylococcus aureus* do not undergo dissolution under similar conditions. Extracts prepared by treating minced pneumonic lung tissue with chloroform, toluene, and saline solution, or the sediment from such

extractions, when added to pneumococci at pH 5.5 to 6.95, dis-
solve the cells, but no action takes place if the reaction is as acid
as pH 4.5. From this observation Lord and Nye concluded: "An
enzyme derived from the bacteria themselves or from the cellular
material may be the cause of the dissolution." Avery and Cullen[38-41]
supported this hypothesis. Autolysates and sterile solutions of
pneumococci, when added to suspensions of pneumococcal cells in
phosphate solutions of known pH previously heated to 60° for
thirty minutes, or 120° for twenty minutes, caused lysis of dead
pneumococci and, to a less extent, disintegration of closely allied
organisms such as *S. viridans*, but they had no effect on *Staphylo-
coccus aureus*. The enzyme was most active in a substrate with a
reaction between pH 6 and pH 8; it was destroyed by heating for
thirty minutes at 60° and was not type-specific in its action. The
bacteriolytic action was proportional to the concentration of en-
zyme. Avery and Cullen expressed doubt whether lysis of pneumo-
cocci under these circumstances was the result of a single enzyme
or the product of the interaction of more than one. The matter was
left undecided by their saying that "whether the enzyme or group
of enzymes concerned in autolysis of pneumococci play any part
in this form of lysis are questions at present undecided."

In 1929, Goebel and Avery[520] were able to state that:

1) Autolysis of Pneumococcus is accompanied by proteolysis, which
results in an increase in amino and non-coagulable nitrogen; 2) autoly-
sis of Pneumococcus is accompanied by lipolysis during which there
is a liberation of ether-soluble fatty acids; 3) when extracts containing
the active intracellular enzymes are added to heat-killed pneumococci,
lysis of the cell occurs and there is an increase in the non-coagulable
and amino nitrogens, comparable to the changes accompanying spon-
taneous autolysis; 4) when extracts containing the active intracellular
enzymes are added to emulsions of the alcohol-soluble lipoids extracted
from pneumococci an increase in the ether-soluble fatty acid occurs.

In spite of the earlier belief of Jobling and Strouse (1913)[680]
that lysis of pneumococci may be independent of ferment action
and the statement of Pauli[1071] that this autolysis is due to the self-

production of peroxide, it would now seem wise to conclude that the self-dissolution of this organism is caused by the action of its own intracellular ferments.

Bile Solubility

The biochemical reaction taking place in the autolysis of pneumococci is also involved in the solvent action of bile on the pneumococcal cell. It was Neufeld[972] who, in 1900, first discovered that bile possessed this unusual property, a property which became of great diagnostic value in differentiating Pneumococcus from Streptococcus and other organisms, and which has since been known as the "Neufeld phenomenon." He found that rabbit bile in a ratio of 1 to 50, or often of 1 to 200 or 300, added to fresh bouillon cultures of living pneumococci, within a fairly wide range of temperature of the fluids, rendered them clear and transparent in the space of a few minutes. The microscope failed to reveal any formed elements, for there was rapid and complete dissolution of the pneumococcal cells. The bile of other animal species and of man, despite considerable variation in the content of bile-acid salts and mucus, exhibited the same property. Bile from rabbits was the most active, and clear, limpid bile was to be preferred to that which was more viscous or cloudy. Its action was not altered by previous heating, but no lysis took place if the cocci had first been killed by heat. Neufeld thought this action was attributable to cholic acid in combination with glycocol and taurin, since glycocol and taurin alone are inactive.

Nicolle and Adil-Bey (1907)[1007] preferred rabbit bile to that of the ox because of its greater solvent action on pneumococci, but better still were sodium cholate and sodium choleate. The authors found that this property was shared, although to a somewhat less degree, by quinolate, taurocholate, glycocholate, and least of all, hypocholate. Bile-solubility was looked upon as a salient character of Pneumococcus.

The advantages of sodium taurocholate over whole bile, claimed

by Levy,[803] were denied by Mandelbaum,[865] who maintained that
while the former dissolved strains of Pneumococcus and *Strepto-
coccus mucosus* as against a negative action on other streptococci,
dissolution was not so complete as that produced by bile. The par-
tial failure of sodium taurocholate may have been due, as pointed
out by Levy, to lack of uniformity in commercial preparations of
the salt. A pure preparation of this bile-salt is to be preferred to
whole bile, because solutions of constant concentration may be
prepared, sterilized, and kept in stock for quantitative or repeated
tests.

Malone[861] would further refine the test by always using the same
strength of sodium taurocholate (10 per cent), keeping the den-
sity of the bacterial suspension uniform, employing cultures from
solid rather than from liquid media, holding both time and tem-
perature constant, and taking precautions that the mixtures are
always alkaline in reaction. For routine identification tests such
refinements seem to be superfluous.

Neufeld at first held the view that only freshly isolated and viru-
lent strains of pneumococci were susceptible to the solvent action
of bile, but Levy,[803] using sodium taurocholate in a 5 to 10 per
cent solution, believed that the method afforded a clear-cut differ-
entiation between all pneumococci and streptococci and other bac-
terial species. It was Levy who, in 1907, with this confirmatory
test, definitely established *Streptococcus mucosus* as a pneumo-
coccus, and called it *Pneumococcus mucosus.*

Neufeld's view concerning the correlation between virulence and
bile-solubility was upheld by Truche, Cotoni, and Raphael[1425] who,
after an experience of several years, reported that high virulence
always accompanied complete solubility and total avirulence went
with insolubility, whereas strains of slight virulence showed partial
or varying solubility. All thirty-one strains of streptococci tested
were insoluble in bile. Malone[861-2] also observed that pathogenic
strains and members of fixed types tended to fall into a sodium
taurocholate soluble group, while the so-called "normal" strains

and cocci of Group IV fell into either soluble or insoluble groups, with some strains intermediate between the two.

Schiemann's* observations did not agree with those of Neufeld. He found completely avirulent strains which were still bile-soluble, as well as mouse-virulent strains which were insoluble. Cocci in the blood and peritoneal exudate of freshly dead mice resisted dissolution, while transitional forms were only incompletely dissolved. Cotoni* described some virulent, typical pneumococci as insoluble. In marked contrast to these results were those of Falk and Jacobson[381] who could find no relation between virulence and bile-solubility, since avirulent strains appeared to be as sensitive to the lytic action of bile as were virulent cultures. Kelly,[702] too, noted a variation in the susceptibility of different strains to lysis, but added that specimens of bile differed in solvent power, and that the presence of sugar in the medium containing the pneumococci had an inhibitory action on the lytic action of bile. Human blood serum, on the contrary, according to Ziegler,[1570] does not interfere with this power.

Differing in their experience from that of Neufeld, Cole and his colleagues[36] found all of several hundred strains of Pneumococcus isolated from lobar pneumonia to be bile-soluble. There are rare exceptions to this rule, an example being the strain of *Pneumococcus mucosus* reported by Dochez and Gillespie.[322] It seems safe to say, with Mair,[552] that "different strains of Pneumococcus show varying sensitiveness to the action of bile, just as they vary in the readiness with which they undergo autolysis in culture, but with a satisfactory technique one is very seldom in doubt as to whether a particular strain should be classed as bile-soluble or not, and strains which have been kept on culture media for long periods retain the property."†

* Quoted by Neufeld and Schnitzer.
† Sellards[1254] also stressed the importance of the characters of the cultures and the alkalinity of the medium in solubility tests. In place of bile Sellards substituted 0.01N to 0.2N solutions of sodium hydroxide, which even in the stronger concentration failed to dissolve streptococci. He thought that old strains of pneumococci were more soluble than young strains.

Harkins[590] contributed the interesting but unexplained observation that the bile of animals in which an artificial cholecystitis had been set up by intracystic injections of organisms other than Pneumococcus (*B. coli communior* excepted), was devoid of any lytic ability. The absence of this property was not due to absorption as shown by the fact that the addition of the infecting bacteria (*S. mitis*, *Staphylococcus aureus*, and *B. coli communior*) to normal bile failed to rob it of this lytic action.

Kozlowski[751] further elucidated the action of bile-solubility by demonstrating that the higher unsaturated fatty acids in bile were even more powerful than cholic acid. The sodium salt of one of these fatty acids with an iodine number of 174, in dilutions of approximately 1 to 50,000, inhibited the growth of pneumococci and, in a dilution of 1 to 5,000, completely killed and dissolved the cells. The soaps were active in higher dilutions and in a shorter time than were the corresponding salts of taurocholic and glycocholic acids. Ziegler[1571] reported that the cytolytic action of sodium dehydrocholate was comparable to the action of sodium taurocholate.

Downie, Stent, and White[327] tested the solvent action of saponin, cholic, dehydrocholic, dehydro-oxycholic, apocholic, and desoxycholic acids, as well as a group of choleic acids which are addition compounds of desoxycholic acid. Of the substances tested, saponin and the sodium salts of all the bile-acids except dehydrocholic and dehydrodesoxycholic acids, brought about lysis. The similarity of the action of the sodium salts of the various choleic acids to that of sodium desoxycholate indicated to the authors that the former owed their activity to the desoxycholic fraction.

Ziegler[1572] found that sodium dehydrocholate and dehydrodesoxycholate would dissolve pneumococci, thus disagreeing with Downie, Stent, and White, who used cultures grown on artificial media. Ziegler employed strains obtained directly from infected animals without any intervening cultivation. He candidly remarked that his paper created more problems than it solved.

In studying the combined action of alkaline oleates and lino-

leates and immune serum on the lysis of pneumococci, Lamar[773] noted that the oleates rendered the cocci more susceptible to serum lysis. In normal serum their action was incomplete, but in immune serum there was no multiplication of the organism and lysis was complete. As an explanation, Lamar suggested that the action of the soap was exerted upon the lipoidal portion of the bacterial cell, through which it was rendered more pervious to serum constituents and brought under their deleterious and dissolving influence.

In another paper, Lamar[774] reported that sodium linoleate and sodium linolenate killed and dissolved pneumococci more rapidly and in higher dilutions than did sodium oleate. Furthermore, in the experiments it was seen that blood serum inhibited the bacteriolytic action of the unsaturated soaps, partly or completely, depending upon the quantitative ratio between serum and soap. Lamar concluded that the action was probably caused in part by the avidity of the unsaturated fatty acids for protein and not wholly by their ability to dissolve lipids.

In 1926, Falk and Yang[385] compared the solvent action of sodium oleate, sodium hydroxide, tribasic sodium phosphate, and saponin on washed pneumococci, and learnt that all these substances in the concentrations tested, with the exception of saponin, were as specific in their action as is bile.

In another communication, Falk and Yang[384] described the influence exerted by certain electrolytes and non-electrolytes on bile-solubility. Chlorides with monovalent cations when present in relatively low concentrations inhibited the solution of washed pneumococci by bile, but in higher concentrations possibly accelerated solution. Chlorides with divalent cations acted in reverse fashion, inhibiting more effectively in high than in low concentrations. Of the anion series tested, sodium hydroxide and tribasic sodium phosphate were cytolytic to pneumococci, whereas dibasic and acid sodium phosphate, sodium sulfate, and sodium nitrate were not. Peptone, gelatin, and ovalbumin appeared to inhibit cytolysis by bile in the same manner as calcium or barium chloride. Falk and

Yang's observation on saponin does not agree with that of Downie, Stent, and White.

Klein and Stone[721] made the interesting observation that while pneumococci were not dissolved by saponin when tested in plain broth culture, preliminary treatment of the cocci with cholesterol rendered them susceptible to complete and rapid lysis by this substance. An excess of saponin inhibited the sensitization by cholesterol and, conversely, an excess of cholesterol inhibited lysis by saponin. The authors thought, on apparently good evidence, that the action of such body fluids as blood and ascitic and pleural fluids in sensitizing pneumococci to saponin lysis was attributable to the cholesterol content. Klein[720] later reported that ergosterol effected a similar sensitization.

In 1930, Neufeld and Etinger-Tulczynska[983] explained more details of the process of bile-lysis. Using bile, sodium taurocholate, and sodium linolenate, it was found that bile and bile-acid salts possessed the greatest solvent action for pneumococci, although soap and bile action were not always parallel. It was further observed that the same antiseptics (one per cent phenol, one per cent formalin, weak ammonium sulfide, acetone, and 0.05 per cent mercuric chloride), which interfere with bile-solubility, in general also inhibited spontaneous autolysis as well as the solution of heat-killed pneumococci by the bacteriolytic enzyme shown by Avery and Cullen[41] to exist in Pneumococcus. Neufeld and Etinger-Tulczynska stated that "the effect of antiseptics would indicate that bile-solubility and action of autolytic ferments are related," but opposed to the concept of parallel bile-solubility and autolysis there is still evidence that the bile-salts have a lytic action of their own, independent of autolytic ferments. The evidence is furnished by the study of Goebel and Avery[520] on autolysis of Pneumococcus. In addition to finding that autolysis was accompanied by definite proteolysis and lipolysis, they observed that while sodium desoxycholate in excess inhibited the action of pneumococcal protease, it did not inhibit the action of the lipase. Goebel and

Avery found, furthermore, that when suspensions of pneumococci were cooled at 0°—a temperature at which the rate of enzyme action is greatly retarded—the organisms went into solution rapidly when sodium desoxycholate was added, and the process was not accompanied by lipolysis or proteolysis. These authors concluded that it did not seem probable, therefore, that the bile solution of Pneumococcus was identical with the phenomenon of autolysis as ordinarily understood and measured. Unpublished work by Dubos would, on the contrary, seem to indicate that bile solution occurs only under conditions when cell ferments remain active.

There can be no doubt, however, that the intracellular enzymes may act as adjuvants in cell dissolution. The action of extracts of living pneumococci in dissolving dead pneumococci (Avery and Cullen[41]) with an increase in non-coagulable and amino nitrogen and in ether-soluble fatty acids (Goebel and Avery[520]) favor this view, a view which has received additional support by the experiments of Wollman and Averbusch,[1535-36] who were able to cause bile solution of killed non-soluble, virulent, and avirulent pneumococci by the addition of a small amount of a living, avirulent culture to the bile-culture mixture. This phenomenon they called *autolyse transmissible.*

Atkin[29] made the original observation that organisms comprising the papillae which frequently arise as secondary growths on autolyzed colonies of pneumococci on serum agar were quite insoluble in bile. It was evident that the organisms of which the papillae consist were devoid of autolysin since they showed no tendency to undergo dissolution or self-digestion even after several weeks of incubation. The organisms thus derived, when subcultured on fresh serum agar, regained autolytic power and at the same time became bile-soluble. Atkin made the statement that "bile solubility of pneumococcus is due to an acceleration of the normal autolytic process by this substance, and that no solution of the organism occurs except in the presence of the autolysin."

Another observation which contributed to the question was that

of Lord and Nye,[835-7] who found that more rapid dissolution of pneumococci occurred in bile than in standard buffer solutions and that lysis proceeded at a more rapid rate when the reaction of the mixture was slightly alkaline. The authors attributed the effect to the more rapid death of the organisms in bile and, hence, to the liberation of a larger amount of enzyme at the optimal hydrogen ion concentration.

Whatever may be the facts regarding the exact nature of bile solution of Pneumococcus, it cannot be gainsaid that this phenomenon and that of autolysis bear striking similarities. Whether or not they are due to entirely separate mechanisms, bile may be regarded as an accelerator of natural autolysis.

Sensitiveness to Germicides and Other Chemical Substances

Pneumococcus is highly vulnerable to the usual antiseptics and germicides. The problem of killing pneumococci and of rendering infectious material harmless is a simple one, and can be dismissed with scant discussion. As an example of the effective concentrations of two common germicides, Schiemann and Ishiwara[1230] gave for the growth-inhibiting strength of phenol and mercuric chloride 1 to 600 and 1 to 100,000, respectively. These figures were obtained by adding a small quantity of a twenty-four-hour broth culture of pneumococci to 5 per cent ox-serum containing varying dilutions of the two substances.*

Simon and Wood[1288-9] investigated the action of a large number of dyes and concluded that the majority of the basic type, especially those of the triphenylmethane series, in a concentration of 1:100,000 in agar were inhibitory for pneumococci as tested by making a stroke culture on the medium. Acriflavine (trypaflavine) acts powerfully both on virulent and non-virulent pneumococci (Schiemann and Baumgarten, 1923). Norton and Davis (1923) found that organisms belonging to the *S. viridans* and Pneumococcus groups were inhibited by dyes to the same extent, so that none could be used for the purpose of differentiating between them.

* Quoted from Browning.[159]

For killing cultures to be used as antigens for the production of active immunity 0.5 per cent phenol and 0.3 per cent formalin are commonly used. Sodium ricinoleate was first used for this purpose by Larson and Nelson,[791] because of its rapid lethal effect on pneumococci and the absence of any impairing action on the antigenicity of the treated cells. Barnes and Clarke[81] determined that sodium ricinoleate and sodium oleate were pneumococcidal in concentrations of 0.04 and 0.004 per cent respectively, which, in conjunction with Lamar's results on the action of sodium lanolate, give us definite figures for these soaps.

In discussing the effect of various chemical substances on the vitality of Pneumococcus, mention may be made of the method developed by Schnabel[1238-9] for measuring such effects. Based on the reducing power of pneumococci, he determined the ability of the organism in contact with varying strengths of the test chemicals to produce significant color changes in methylene blue, litmus, neutral red, indigo blue, and other dyes. Since the reducing power of pneumococci is a variable one, this factor must be carefully controlled by proper observance of time, temperature, and hydrogen ion concentration. The method enabled Schnabel to study both the inhibiting and the sensitizing action of quinine, optochin, mercuric chloride, formaldehyde, phenol, and silver nitrate on Pneumococcus. The method holds possibilities for the study of drug-fastness of the coccus and Schnabel claimed that by the use of this technique he could determine the presence of optochin in a 1 to 1,000,-000 dilution and that it can be applied to the quantitative measurement of the amounts of analogous substances in solution.

The discovery by Morgenroth and Levy[926] that quinine and some of its derivatives, especially optochin, possess bactericidal powers for Pneumococcus, both *in vitro* and *in vivo*, has led to investigations on the therapeutic effect of this class of substances on pneumococcal infection. The results of the studies will be discussed in the chapter on Chemotherapy.

To summarize our knowledge of the bactericidal action of vari-

ous chemicals upon Pneumococcus, it suffices to say that no sub-
stance or class of substances have as yet been found which exert
any selective lethal action on the cell with the exception of bile-
acids and certain derivatives of quinine.

BIOCHEMICAL FEATURES

*The behavior of Pneumococcus toward proteins, fats, and carbo-
hydrates; acid production; oxidizing and reducing action; and
the formation of peroxide, methemoglobin, hemolysin, hemotoxin,
and alleged endotoxin.*

THE pneumococcal cell is charged with a complement of en-
zymes capable of destroying it as well as certain extracel-
lular substances upon which they are allowed to act. The living or-
ganism and sterile extracts made from it can digest proteins, split
lipids, and invert and ferment carbohydrates.

Proteolysis, Lipolysis, and Carbohydrate Fermentation

PROTEOLYSIS

Rosenow[1169] was probably the first to express the opinion, based
on experimental evidence, that Pneumococcus contained a proteo-
lytic enzyme capable of splitting its inherent protein into a highly
poisonous substance and to demonstrate the proteolytic action of
Pneumococcus. Extracts of virulent pneumococci made in sodium
chloride solution and filtrates of broth cultures hydrolyzed the
proteins contained in heated ascites-meat broth and, to a lesser
degree, those of heated serum. The extracts did not attack egg-
white or pure casein. The enzyme appeared to be more resistant to
heat and to long standing in broth filtrates than in salt solution.
Heating at 60° reduced the enzymatic action of the filtrates by 50
per cent, and almost completely destroyed the activity of the saline
extracts.

In 1920, Avery and Cullen[38] gave a still more detailed demon-
stration of the proteolytic action of Pneumococcus. By dissolving
living cocci in bile or sodium cholate, or by allowing them to auto-

lyze under alternate freezing and thawing in phosphate solutions
with a reaction of pH 6.2, or by filtering sterile broth cultures, the
authors obtained cell-free extracts capable of hydrolyzing casein
and fibrin but not albumin and gelatin. The extracts digested pro-
teoses and peptones even more rapidly, converting them into pep-
tides and amino acids. The enzyme or enzymes were designated by
the authors as protease and peptonase, although it was stated that
these names might merely represent different activities of the same
ferment. The activity of the enzyme was favored by a reaction
of pH 7.0 to 7.8, but was suspended at pH 5.0. The enzyme,
however, was not destroyed by relatively short exposure to this
acid reaction, since its activity was restored when the pH of the
solution was readjusted to 7.8. The rapidity of hydrolysis was
proportional to the concentration of enzyme. Heating diminished
the digestive action, which was lost after ten minutes' exposure to
100°. The ability of active extracts to digest the protein of heat-
killed pneumococci was later shown by Goebel and Avery[520] by
demonstrating an increase in the non-coagulable and amino nitro-
gen after contact of the extract with the killed cocci.

LIPOLYSIS

The presence of an esterase in the pneumococcal cell has also
been demonstrated by Avery and Cullen.[39] Extracts of pneumo-
cocci, prepared in the manner employed for obtaining proteases,
split tributyrin. Heating for ten minutes at 70° destroyed the
activity of the lipase. While bile and bile-salts accelerated lipoly-
sis, they were not essential to the reaction, since pneumococcal ex-
tracts prepared in other ways exhibited the same enzymatic ac-
tivity. In the study of Goebel and Avery it appeared that on the
addition of extracts containing the active intracellular enzymes of
the pneumococcal cell to the ether-soluble lipids of pneumococci,
an increase in ether-soluble fatty acid occurred. Falk and Mc-
Guire[386] recently reported that strains of Pneumococcus, Types I
and II, in suitable broth, hydrolyzed phenylacetate, glyceryl tri-

acetate, methyl n-butyrate, and benzylacetate. While different degrees of hydrolysis were shown for the four esters, the comparative ratio of the action of the two types of organisms on these esters was of the same order in different lots of broth, although the absolute values were not the same. As a rule, Type I organisms showed a greater action than those of Type II, except in the case of phenylacetate.

CARBOHYDRATE FERMENTATION

Important from a practical standpoint is the content in Pneumococcus of carbohydrate-splitting enzymes, since the selective action of the latter serves in the bacteriological differentiation of this species from other species and genera of the family *Coccaceae*.

Invertase, amylase, and inulase were first demonstrated in Pneumococcus by Avery and Cullen (1920).[40] Finding that bile solutions of pneumococci were unsuited to the purpose, the authors used sterile extracts made by alternately freezing and thawing suspensions of cells in balanced phosphate solutions at a pH of 6.2. The enzymes were active within the limits pH 5.0 to 8.0, with an optimum of 7.0. The acid death-point of pH 5.0 may be reached in carbohydrate media, even when buffered with phosphates, if the content of glucose exceeds 0.3 per cent (Avery and Cullen, Lord and Nye[829]). These authors agreed in ascribing carbohydrate fermentations to intracellular or endoenzymes. The saccharolytic ferments of Pneumococcus have a lower thermal death-point than the protease or lipase, being destroyed at 55° in ten minutes.

The enzymatic preparations freed of living cells hydrolyze sucrose, starch, and inulin but, strange to say, fail to ferment glucose, although in culture media the cleavage of this sugar into acid is a definite and constant activity of the growth of Pneumococcus. According to Hewitt,[643] the breakdown of glucose by Pneumococcus is a less complex process than in the case of many other organisms. Of the glucose constantly disappearing from such cultures, about 78 per cent was recovered by Hewitt as lactic acid.

There appeared to be marked and characteristic divergences in the behavior of different types of pneumococci toward this sugar, the variant RIII being first and SI second in order of glycolytic activity. It was Hewitt's belief that inorganic phosphorus played an essential part in the fermentation of glucose and that this part was independent of its buffering action. The full benefit to Pneumococcus from phosphates was only obtained when media containing these salts were sterilized by filtration and not by autoclaving.

The cleavage of maltose and lactose, unlike that of glucose, is due respectively to a maltase and a lactase in the pneumococcal cell (Fleming and Neill[452]), while Neill and Avery[954] have demonstrated a raffinase.

A significant and distinctive enzymatic property of Pneumococcus is its ability to ferment inulin, a polyose from dahlia bulbs. It was Hiss[649] who first in 1902 discovered that, by means of this property, it was possible to differentiate pneumococci from the closely related members of the tribe *Streptococcaceae*. Serum-water containing one per cent inulin supported growth of Pneumococcus, resulting in the development of acidity and coagulation, while streptococci, although growing, failed to form appreciable acid or to coagulate the medium. It was the use of this method which, with other biological characters, indicated that the so-called *Streptococcus mucosus* was in reality a pneumococcus.

Duval and Lewis[342] overcame the variations encountered in various batches of serum-water by using inulin broth. They found that in its fermentative ability *Streptococcus mucosus* closely resembled Pneumococcus, but decided that the inability to ferment inulin did not necessarily exclude an organism from the species, since some pneumococci failed to show this character. Dochez and Gillespie[322] described a strain of *Pneumococcus mucosus* that did not ferment inulin. Berry[106] also observed the pneumococcal characters of *S. mucosus*, and her results agreed with those of Duval and Lewis in that she encountered other strains of pneumococci that failed to attack inulin, as well as variations in this respect

in strains that had undergone changes in morphology and viru-
lence induced by longer or shorter cultivation on artificial media.
Berry drew attention to the lack of uniformity in the serum-water
medium and in commercial preparations of inulin. With a reliable
lot of inulin serum-water, however, some non-inulin-fermenting
strains acquired or regained this fermentative property after
animal passage.

In a more recent paper, Berger and Silberstein[103] reported the
results of a study on strains of pneumococci, of the "B" modifica-
tion* of this species, and of green-producing and hemolytic strep-
tococci. The hemolytic streptococci; none of which affected inulin,
were the only group with constant behavior toward this carbo-
hydrate. Of the typical pneumococci, four strains showed redden-
ing without coagulation of the medium and two had no inulin-fer-
menting power. These two strains were found to be modification
"A"* when tested with optochin. Of nine strains of modification
"B" obtained from Pneumococcus, but behaving otherwise as green
streptococci, two retained the inulin-fermenting property. Of
thirty green streptococci, five gave marked inulin fermentation,
while four others showed slightly positive reactions. Other authors
to report strains incapable of fermenting inulin among the pneu-
mococci are Hiss; Park and Williams; Levy; Avery; and Bürger
and Ryttenberger.†

In addition to inulin, the polysaccharide, glycogen, is equally
susceptible to the saccharolytic action of Pneumococcus. First re-
ported by Hiss,[649] this reaction has been quantitatively studied by
Barnes and White,[85] who compared the fermentative reactions of
strains of Type I, II, III, and V pneumococci on glucose, inulin,
mammalian glycogen, and glycogen obtained from scallops. The
authors decided that, on the whole, the four type strains used fer-
mented glycogen to essentially the same degree as they did glucose
and inulin.

Pneumococcus attacks still other carbohydrates, but no specific

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* To be described in Chapter V.   † Quoted by Neufeld and Schnitzer.

enzymes have been demonstrated for these reactions. In addition to glucose, galactose and levulose are actively fermented with the formation of lactic acid, as well as the trisaccharide, trehalose, and the glucoside, salicin. The fermentation of alcohols is much less marked. Slow acid production takes place in media containing glycol, glycerol, and erythritol; some strains slowly produce acid from mannitol, but there is no action on dulcitol or sorbitol. The pentoses, arabinose and xylose, are slowly attacked (Mair[552]). Zo- zaya[1586] added levan, obtained from *B. mesentericus* and *B. sub- tilis*, to the list of substances fermentable by Pneumococcus. Dex- tran, obtained from *Leuconostoc mesenteroides* was not affected. Although the change in levan was less than that in inulin, the acidity in both cases seemed to be mainly due to lactic acid.

Acid Production

Supplementing the brief mention that has already been made of the formation of acid from saccharides by Pneumococcus, some of the factors that condition that reaction are as follows: The amount of sugar present in the medium determines the degree of acidity attained. If sufficient carbohydrate is present, growth ceases at an acid reaction of pH 5.0. If there is less than 0.4 per cent of sugar, growth ceases at a lower hydrogen ion concentra- tion, apparently because of exhaustion of carbohydrate. If no carbohydrate is present save that extracted from the meat from which the broth is made, growth initiated at pH 7.8 ceases at about pH 7.0. If the reaction of bacteria-free filtrates of plain broth cultures in which growth has ceased is readjusted to pH 7.8 and the medium reinoculated with Pneumococcus, no growth oc- curs unless carbohydrate is added. However, if bacteria-free fil- trates of dextrose-broth cultures in which growth has ceased are readjusted to pH 7.8 and reinoculated with Pneumococcus, growth is resumed. Cultures of Pneumococcus, with maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin give identical re- sults in the rate of reaction change and the final hydrogen ion

concentration (pH 5.0) attained. Such are the conclusions of Avery and Cullen[37] in their 1919 study. Jones,[683] a year later, explained the failure of these authors to produce an acidity greater than a final pH of 7.0 by the fact that they did not add glucose to their beef-infusion medium, but it would seem that this failure had already been accounted for in other parts of their report.

The degree of acid production by pneumococci appeared to Gundel[569] to be correlated with pathogenicity. He grew strains isolated from human mouths in lactose bouillon (pH 7.0) and in this medium noted only a slight acid formation by pathogenic strains, whereas non-pathogenic pneumococci produced marked acidity with transition forms between the extremes. No confirmation of Gundel's results appears to have been published.

The addition of normal human serum to suspensions of living pneumococci in isotonic bouillon, while preventing cell dissolution, does not inhibit acid production (Lord and Nye[834]). In rabbit serum, according to Bordet,[139-40] the pneumococcidal hydrogen ion concentration is pH 6.2 for Types I, II, and III, and here the acidity is due to formic and acetic acids.

Oxidation and Reduction

Pneumococcus, under varying degrees of oxygen tension, manifests the phenomena of oxidation and reduction. With the activities of its oxidases are correlated, either directly or indirectly, synthesis and hydrolysis of pneumococcal protein and carbohydrates, changes in the hydrogen ion concentration, the formation of peroxide, the conversion of hemoglobin into methemoglobin, the lowering of the oxygen capacity of blood cells. and the death of the organism itself.

METHEMOGLOBIN PRODUCTION

In 1913, Butterfield and Peabody,[194] seeking an explanation for the reduction of the O_2 capacity of the blood in human lobar pneumonia, discovered that upon incubating pneumococcal cultures

with washed rabbit corpuscles, there ensued a diminution of the oxygen-carrying capacity of the cells, owing to the formation of methemoglobin, or some derivative of hemoglobin with identical optical constants for these regions of the spectrum. The substance which induced this change was also present in sterile filtrates of autolyzed cultures. The authors concluded that the mechanism of the reduction of the O_2-carrying capacity of the blood in human lobar pneumonia was, in part at least, of the same nature. In rabbits with a severe experimental bacteriemia it was found that the O_2-combining power of the venous blood fell progressively up to the time of death. Coincidentally, there was an even more marked fall in the O_2 content of the arterial blood. The changes in the blood of infected animals appeared to be analogous to those seen when Pneumococcus was grown in blood *in vitro*, the oxygen-carrying capacity of the cells in both instances being due to the conversion of hemoglobin into methemoglobin.

In the next year (1914), Cole[253] reported conclusions which, because of their importance, may be summarized here: Pneumococci in contact with hemoglobin transform this substance into methemoglobin, and the reaction occurs only when the pneumococci are living; it is not induced by the culture fluid or by extracts of the bacteria, differing in this respect from the results of Butterfield and Peabody. The reaction does not occur when hemoglobin is added to an emulsion of washed pneumococci in salt solution. However, if minute traces of dextrose be added to the mixture, the transformation occurs quickly. Dextrose may be replaced by any one of a number of other sugars, and also by some organic compounds, if the latter are added in large amounts. Certain other organic substances were unable to replace dextrose, but it was impossible to determine any special molecular configuration upon which this property depends. The formation of methemoglobin by pneumococci probably resembles the changes induced in the blood by such chemical substances as amino phenol. It seems not unlikely that the transformation is always a reaction of oxidation. In the

presence of reducing agents, the latter are first oxidized, the action occurring more readily in the presence of oxyhemoglobin. In some instances an alternate oxidation and reduction of the transformative agent occurs, so that the reaction is continuous. The effect of the presence or absence of free O_2 on the changes in blood pigment induced by chemical agents of known composition suggests that the reaction with Pneumococcus follows similar lines. The reaction does not occur in the absence of O_2. If the free O_2 be first removed and then replaced, the reaction takes place more rapidly than if the O_2 had not been removed. The presence of free O_2 in excess slightly delays the reaction, possibly because of the inhibition of the reduction process which forms the first part of the reaction.

In 1924, Morgan and Neill,[916] as a result of their experiments, concluded that:

Sterile filtrates of aerobic cultures of Pneumococci containing H_2O_2, were capable of converting catalase-free solutions of oxyhemoglobin into methemoglobin. In catalase-containing solutions of hemoglobin from laked corpuscles, the actual methemoglobin-forming system of Pneumococcus involves a labile constituent of the bacterial cell, which is itself susceptible to oxidizing agents and may be rendered inactive if exposed to peroxide or similar substances previous to its introduction into oxyhemoglobin solutions. The activity of this function, in the case of sterile filtrates, depends, therefore, upon the liberation of cell constituents into the medium and upon the protection of those cellular substances from the oxidizing agents which are formed when Pneumococcus cultures are freely exposed to air. When these cultural conditions are fulfilled, sterile culture filtrates of Pneumococcus convert oxyhemoglobin into methemoglobin independent of the presence of blood catalase.

In 1921, Stadie,[1311] in studying the blood changes in pneumococcal infections, found that methemoglobin disappeared rapidly from the blood stream, whether introduced by injection or formed within the circulation by pneumococci or by the action of chemicals (potassium ferricyanide and sodium nitrate). He concluded: "In

the occasional cases of pneumonia which show a decrease in the oxygen capacity of the blood, the decrease is probably due to the formation of methemoglobin. The latter is removed from the circulation, however, as rapidly as it is formed, so that it can seldom be detected even qualitatively, and is probably never the cause of cyanosis." The practical impossibility of demonstrating methemoglobin in the circulating blood was also experienced by Schnabel.[1239]

The explanation of the phenomenon of methemoglobin production is of importance not only in so far as this special reaction is concerned, but also because it suggests a mechanism by which pathological effects may be produced by bacteria which apparently elaborate no soluble toxin.

PEROXIDE FORMATION

The work of McLeod and Govenlock,[885] of McLeod and Gordon,[881-3] and of McLeod, Gordon, and Pyrah[884] was confirmatory of Cole's results and amplified our knowledge of the property possessed by pneumococci to form peroxide during growth. The authors ascribed the greenish or yellowish discoloration produced on heated blood media to an accumulation of peroxide, showed that the death of pneumococci in cultures was brought about by an excess of the same product, and demonstrated that the substance was not organic peroxide but hydrogen peroxide. It was not formed in cultures deprived of oxygen, nor in cultures that contained abundant catalase.

Penfold[1077] disclosed another manifestation of the oxidizing action of pneumococci. According to him, all pneumococci and probably all streptococci acting on certain aromatic amines, notably aniline, benzidine, and the toluidines, produce pigment. When pneumococcal and streptococcal cultures were grown on citrated horse-blood agar containing benzidine, the colonies appeared black by transmitted light and metallic blue by reflected light with dark discoloration extending far into the medium around the colony. This pigment production from the amines is a peculiarity shared

only by pneumococci and streptococci and, according to Penfold, is due to the peroxide produced by these organisms.

In the next year, Felton[395] studied the oxidase reaction of bacteria by means of the oxidation of p-amino leuco-malachite green, and found that the conditions most suitable for the reaction were the presence of a slight amount of hemoglobin and of dextrose, an acid reaction, the presence of fresh serum heated for thirty minutes at 56°, and a plentiful supply of oxygen. In order of decreasing suitability for promoting oxidation were rat, guinea pig, rabbit, horse, human, cat, and chicken serum. Of the organisms tested by Felton, only Pneumococcus, *S. viridans*, and *S. haemolyticus* exhibited oxidative power, the first named giving the most intense reaction. That this power was not an exclusive character of the three species was shown in the same year (1923) by McLeod, Gordon, and Pyrah,[884] who added *B. bulgaricus* and *B. acidophilus* to the group of peroxide-producing bacteria.

Directly opposed to the conclusions of McLeod and Gordon and of Cole, but somewhat analogous to those of Felton, were the views of Barnard and Gowen.[80] These two authors discounted the possibility that the green disintegration product of hemoglobin referable to the growth of pneumococci is methemoglobin, since methemoglobin will not exist in the presence of hydrogen peroxide. A green pigment, identical in all respects with that produced by the growth of pneumococci on hemoglobin, was prepared by Barnard and Gowen by the action of peroxide and certain nitrogenous compounds on blood pigment. A chemical study of this artificial green pigment indicated to the authors that it was not methemoglobin but a xanthoprotein compound. Without more complete information it is impossible to judge the correctness of the claim, but studies from the Hospital of the Rockefeller Institute seem to prove the case for methemoglobin. It is, however, possible that the appearance of xanthoprotein may be an additional effect of peroxide.

Avery and his colleagues published a series of papers that mate-

rially amplified our knowledge of this phenomenon of peroxide production by bacteria. Avery and Morgan[52] noted the early appearance of peroxide in cultures of pneumococci and of non-hemolytic streptococci, with a somewhat retarded development in cultures of some but not all strains of *S. haemolyticus* and *S. mucosus*. Peroxide was not detected at any time during the growth of two strains of *Staphylococcus aureus*. The factors influencing peroxide formation were free access of air, and the absence of a catalase, peroxidase, or other catalyst capable of decomposing H_2O_2. Under favorable conditions peroxide production continued during the logarithmic phase of growth and persisted for at least six to twelve days. According to Avery and Morgan the aerobic growth of anaerobic bacteria in broth containing sterile, unheated plant tissue may be related to the action of oxidizing-reducing systems of plant tissue in the destruction of toxic peroxides formed during bacterial growth. These assumptions were supported by subsequent experiments by Avery and Neill,[55] in which it was found that anaerobically grown pneumococci rapidly form peroxide on exposure to molecular oxygen. The peroxide-forming properties of pneumococci varied with different strains and with the age of the cells, and were active under conditions of reaction and temperature that did not permit active cell growth and multiplication—a range of pH 5.0 to 8.5—and at lower temperatures than those that favor growth.

Avery and Neill,[56] employing sterile, filtered extracts prepared by freezing and thawing pneumococcal cells or by dissolving them in bile, proved that the peroxide-forming ability of Pneumococcus is a function not dependent upon the presence of living, intact cells. Sterile extracts of unwashed pneumococci promptly formed peroxide on exposure to air; its formation was almost as active in extracts aerated at $2°$ as in those exposed to air at room temperature. The optimal zone was on the alkaline side of pH 6.0, but peroxide was detectable in the range pH 5.0 to 9.0. The peroxide-

forming activity of the cells was gradually diminished by pro-
longed exposure to 55° and was destroyed by heating for five min-
utes at 65°. Moreover, cells and extracts of cells thoroughly
washed before extraction with salt or phosphate solution showed
no peroxide-producing activity, but such extracts could be acti-
vated by the addition of cell washings, yeast extract, or muscle
infusion.

In a subsequent paper, Avery and Neill[57] reported that sterile
broth extracts of unwashed pneumococci, entirely free from living
or intact cells, actively reduced methylene blue, whereas sterile ex-
tracts of washed pneumococci in phosphate buffer solution were
unable by themselves to reduce the dye. As in the case of peroxide
formation, the reducing action was restored by the addition of
meat infusion or yeast extract to extracts prepared from washed
cells. Platt[1097] reported that meat extract augments the amount of
peroxide formed as do also lactic acid and glucose. Gelatin, on the
contrary, delays its formation, and in gelatin broth no appreciable
amounts of peroxide are present until near the end of the period of
logarithmic increase of bacteria.

The system or systems responsible for methylene blue reduction
are destroyed by exposure to temperatures practically identical
with those which had previously been found to destroy the per-
oxide-forming activity of the same extracts. Avery and Neill[57] sug-
gested that peroxide formation and methylene blue reduction by
pneumococcal extracts are functions of the same or closely related
systems, the particular reaction induced depending upon whether
molecular oxygen or methylene blue serves as hydrogen acceptor
or oxygen donator.

In 1930, Hewitt[641] supplied further confirmatory data concern-
ing the phenomena of oxidation and reduction by pneumococci and
streptococci. In aerobic cultures of both species there is a rise in
potential after the logarithmic phase of growth, while in the case
of diphtheria bacilli and staphylococci, the potential remains at a

low level long after the cessation of active cell proliferation. In a second paper, Hewitt[642] reported the use of liver, blood, or bacteria in culture media as a means of inhibiting peroxide formation and of permitting the study of other oxidation-reduction phenomena. In aerated cultures under these conditions, the potential fell to much lower levels and growth was much more luxuriant. In similar cultures without catalase the potential first declined, then rose to the level at which peroxide can be detected chemically. In the presence of catalase, however, the potential fell, then rose very slowly, and finally reached the level corresponding to peroxide accumulation only after the effective catalase had been destroyed by the active oxidizing system. If fresh catalase was then added an immediate drop in potential occurred.

Lieb[812] did not agree that the oxidation phenomenon had to do with the formation of hydrogen peroxide but thought that it was due to another mechanism operated by an unknown oxygen-carrying factor, possibly a ferment, acting in the same oxygen concentration as hydrogen peroxide. Such a conclusion would appear to be no more than conjecture.

Pauli's work (1927–8)[1071] was more or less a repetition of that of the American school. He explained the fact that Pneumococcus rapidly undergoes autolysis in cultures by assuming that lysis was due to the production of hydrogen peroxide in the absence of catalase. When he grew pneumococci, respectively, in the presence of air, under an atmospheric pressure of thirty millimeters of mercury, or under complete anaerobic conditions, the cells grew well, but autolysis took place rapidly in the first, slowly in the second, and was still absent in the third after a month's incubation. Pauli ascribed the advantage of adding any of the many growth-stimulating substances to culture media to the content of catalase which decomposes hydrogen peroxide at the time of its formation.

Another effect of the oxidizing agents formed when sterile extracts of pneumococci are exposed to air is the destructive action

they exert on the saccharolytic enzymes, sucrase, raffinase, inulase, and amylase, of the cell. Aerated extracts, however, have no inactivating effect on pneumococcal protease or lipase.

The relative resistance of the enzymes of Pneumococcus to heat is consistent with their resistance to the action of hydrogen peroxide. Neill and Avery[953] found, furthermore, that sterile extracts of autolyzed anaerobic cultures of pneumococci contained much higher concentrations of active endocellular enzymes than did filtrates of autolyzed aerobic cultures. The difference may be explained by a destruction of the formed enzymes by oxidative reactions analogous to the destruction observed during oxidation of sterile broth extracts of unwashed pneumococcal cells. The more complete destruction of enzymatic activity in autolyzed aerobic cultures than in oxidized sterile cell extracts is probably due to the longer exposure to oxidation products. More recently Finkle (1931),[440] in a study of the metabolism of pneumococcal variants, confirmed the fact that oxidation may have an inhibitory effect on sugar fermentation. Under anaerobic conditions saccharolysis is approximately the same for organisms of Types I, II, and III, while, under aerobic conditions, only Type I cells are capable of this action. The difference may be explained by the relatively feebler respiratory capacity of Type I organisms. The capacity of these cells is only 56 per cent of that of Type III pneumococci, which in turn is 71 per cent of that of Type II strains.

In a later paper, Neill and Avery[956] reported further details of the oxidation-reduction system of Pneumococcus. The thermostable components of the oxidation-reduction system were still active in oxidized extracts, while the thermolabile cellular constituent was destroyed not only by products formed during oxidation, but also by molecular oxygen itself. The labile component of the system was more susceptible to the action of reagent hydrogen peroxide than any other known intracellular substance of pneumococcal origin. Neill and Avery designated the thermostable substances as

RH, the actual source of the oxidizing agents, and the thermo-labile cellular constituent as C, serving as a catalyst in accelerating the reaction, which they formulated thus:

$$1)\ C + R\text{H} + O_2 \rightarrow \text{oxidizing agent}$$
$$2)\ \text{Oxidizing agent} + C \rightarrow \text{inactive } C.$$

RH then represents substances that are not present in Pneumococcus after thorough washing; they are relatively stable, resisting boiling for prolonged periods, and are present in water or alcohol extracts of muscle, yeast, and vegetable tissue. C represents a labile cellular component, inactivated in ten minutes at $65°$. By itself it is non-reactive with O_2 and possesses no reducing power, being apparently catalytic in nature.

Neill[948] observed that both living pneumococci and sterile extracts prepared from unwashed cells were capable of oxidizing hemoglobin to methemoglobin, and that the action was a reversible one, the equilibrium being shifted in either direction by regulation of the oxygen tension. Neill[947] also found quantitative evidence of the reduction of methemoglobin to hemoglobin by sterile animal tissues, such as kidney, testicle, and liver from an exsanguinated rabbit. He concluded, therefore, that "the accumulation of large amounts of methemoglobin in the circulating blood is probably an indication not only of the formation of large amounts of this pigment, but also of a poisoning of the normal reducing mechanism of the animal tissues." This statement is difficult to reconcile with that of Stadie (p. 73, *ante*) that in man, at least, methemoglobin is removed from the circulation as rapidly as it is formed.

In another note, Neill[949] added that in the presence of air the oxidizing activity of pneumococci was much stronger than their reducing activity in the absence of air, while the reverse was true for anaerobic bacteria. From subsequent studies, Neill[950] was able to elaborate the graphic representation of the oxidation-reduction reaction to include the production of methemoglobin:

$a)$ $\begin{cases}
\underbrace{C}_{\substack{\text{Thermolabile} \\ \text{cellular} \\ \text{substance}}} \quad + \quad \underbrace{RH}_{\substack{\text{Thermostable} \\ \text{easily oxidized} \\ \text{substance}}} \quad \underbrace{+ \, O_2 \to \quad ROOH}_{\text{Oxidizing agent}} \\
\text{or} \\
C \quad\quad + \quad\quad 2\,RH \quad\quad\quad + O_2 \to 2\,R + H_2O_2
\end{cases}$

$b)$ $\quad\quad\quad ROOH \text{ (or } H_2O_2) + Hb \to MetHb$
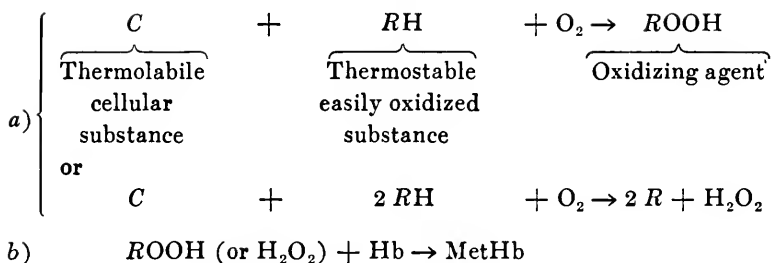
For comparison, Neill developed another formula to represent the process of methemoglobin formation during the auto-oxidation of other substances:

$$a) \begin{cases} RH + O_2 \to ROOH \\ 2\,RH + O_2 \to 2\,R + H_2O_2 \end{cases}$$

$$b) \; ROOH \text{ (or } H_2O_2) + Hb \to MetHb$$
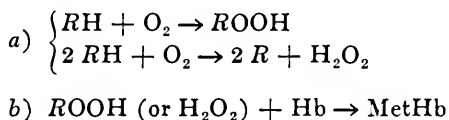
and for the spontaneous formation of methemoglobin in pure hemoglobin solutions, in which the iron of the hemoglobin may serve as a catalyst, as:

$$\text{Hemoglobin} + x\,(O_2) \to \text{Methemoglobin}$$
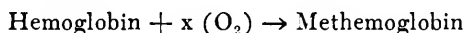
The reactivation of the bacteriolytic activity of oxidized pneumococcal extracts, according to Neill, Fleming, and Gaspari[958] appears as still another effect of the oxidation-reduction phenomenon. When oxidized and therefore inactive pneumococcal extracts were treated with bacterial reducing agents (anaerobic bacteria), the extracts regained their lytic power, and showed a species-specificity which argues for their identity with the original bacteriolytic agent. It was held that this agent is an integral part of the pneumococcal cell, and that its action is reversible and susceptible of being represented by a graphic formula similar to those already given.

Further evidence concerning the reversibility of the action involved in the transformation of oxyhemoglobin to methemoglobin

was supplied by the reports of Schnabel,[1238] and of Schnabel and Ninamiya.[1243] Although few new facts were given in these papers, the authors found that the activity was not lessened by the addition of high concentrations of optochin or sodium glycocholate to broth cultures, while, on the other hand, the alkaline reaction and reducing power of blood serum and of tissue cells interfered with its operation.

Another effect of oxygen consumption by Pneumococcus is the relation between respiration and virulence as described by Sevag and Maiweg,[1258] who presented the following facts in support of their theory: Virulent pneumococci on being transformed into the avirulent forms consume much larger amounts of oxygen than do the parent organisms, but this gain in activity is only temporary. After a time, the avirulent organisms degenerate into forms which consume very much less oxygen than do either the virulent or the avirulent cells recently derived from the parent strain.

The phenomenon of oxidation-reduction by Pneumococcus is given here in considerable detail because of its important bearing on the metabolic process of the cell and because of the thoroughness with which this activity has been studied. It involves the life of Pneumococcus; it affects by inhibition or destruction the lytic and synthetic ferments of the bacterial cell; it is responsible for the conversion of the vitally necessary hemoglobin into methemoglobin and for the lessened oxygen-carrying capacity of the red blood corpuscles; it may play a part in determining virulence of a strain; and it is undoubtedly concerned in the production of hemotoxin and hemolysin and possibly of other substances harmful to the human economy.

Hemolysin and Hemotoxin

Pneumococcus, upon lysis, whether natural, or artificially induced, yields a substance or principle that is actively hemolytic for sheep, guinea pig, and human erythrocytes. The substance is labile, much of its activity is lost on passing through a filter, and

it is destroyed by the action of trypsin. In its properties the hemolysin corresponds to the substance contained in the lytic extracts of pneumococci that cause the death of guinea pigs on intravenous injection. Its activity is prevented by the presence of minute amounts of cholesterol. Following the injection of autolysates into rabbits and sheep, the serum of the animal acquires increased power of inhibiting the hemolytic action and therefore Cole (1914),[252] whose conclusions these are, believed that the hemolysin has antigenic properties; that it is not simply a product of autolysis but undoubtedly exists preformed in the pneumococcal cell; it is not given up to the surrounding fluid so long as the bodies of the cocci are intact and, accordingly, is to be considered as a hemolytic endotoxin.

Previous as well as subsequent to the work of Cole, there have been isolated observations of a zone of hemolysis about the colonies of pneumococci on blood agar. Libman,[311] in 1905, demonstrated an organism, probably a pneumococcus, isolated from the blood of a pneumonia patient on the fifth day of the disease, which on blood plates produced a peculiar hemolysis. Later (1922) Hewitt and Famulener[640] described the production of a zone of hemolysis immediately around a colony on blood agar of an organism, beyond doubt a pneumococcus, isolated from the blood in a fatal case of septicemia with meningitis following mastoiditis. From their study, Hewitt and Famulener inferred that pneumococci of all serological groups, under certain cultural conditions, may hemolyze human erythrocytes, and that this property apparently is not influenced by the reaction of the medium within the growth limits of the organism nor by prolonged refrigeration of the developed colonies on blood-agar plates. The authors also expressed the opinion, later substantiated by Avery and Neill, that the hemolysin was an intracellular product liberated from the autolyzed organisms, which diffuses from the colony into the surrounding blood agar. In contradistinction to the hemolysis of *Streptococcus haemolyticus*, Hewitt and Famulener called the pneumococcal effect "pseudo-hemolysis."

Takami[1372] described a zone of hemolysis about the colonies of all the sixty strains of pneumococci plated on blood agar. This zone was separated from the periphery of the colony by a greenish zone; it appeared on standing at room or ice-box temperature and in time might spread over the whole plate.

Avery and Neill[58] made further studies on this property of Pneumococcus. Extracts of pneumococci prepared in broth, or from washed pneumococci made with phosphate solution and filtered through Berkefeld candles, were actively hemolytic for rabbit erythrocytes. The hemolysin was destroyed by ten minutes' heating at 55°. The conclusions of the authors were:

> When pneumococcus extracts are exposed to air, the hemotoxin is destroyed only in those extracts capable of undergoing auto-oxidation. The active agent responsible for this destruction is probably a peroxide formed by the union of molecular oxygen with some other easily oxidizable constituents of the extracts.

In another study, Neill[951] mixed pneumococcal extracts, in which the hemotoxin had previously been inactivated by oxidation, with *B. coli* and anaerobic *Bacillus T*, sealed and incubated the extracts thus treated for several hours. Titration of the bacteria-free supernatant fluid obtained by centrifuging this mixture showed that the inactive oxidation product of pneumococcal hemotoxin was reconverted to an actively hemolytic substance by the action of those bacteria, which were not themselves hemolytic. The reduction was also accomplished by sodium hyposulfite. The active lysin, or hemotoxin, produced by the reduction of the inactive oxidized extracts was shown to be identical with the original active hemotoxin, since it possessed the same degree of thermolability and was neutralized by the same specific antibody. Pneumococcal hemolysin or hemotoxin is, therefore, not an artificial cleavage product but a natural substance originating within the cell. Normal serum, egg albumen, cholesterol, and peptone, according to Weiss,[1509] inhibit hemolytic activity.

Returning to the original observation of Cole that active or reduced pneumococcal hemotoxin is antigenic, Neill[952] in 1927 reported that "a neutralizing antibody may be produced by immunization with the hemolytically inactive hemotoxin present in oxidized solutions as well as by immunization with the active toxin. The antibody is a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci." Neill also showed that the hemotoxin is independent of the bacteriolytic enzyme present in pneumococcal cells. In the next year, Cotoni and Chambrin[282] confirmed the data reported by Cole and by Avery and Neill, and de scribed a simple method for the quantitative measurement of the hemolytic power of Pneumococcus. While Cotoni and Chambrin were able to make satisfactory determinations on a number of strains, they reported that the quantity of hemoglobin liberated did not follow the law of multiple proportions and that strains varied in their hemolytic action. To the authors there appeared to be no difference in the susceptibility of the blood cells of man, rabbit, guinea pig, cow, pig, horse, or sheep to pneumococcal hemolysin. They also gave a method for titrating the antihemolytic power of immune serum.

Sickles and Coffey[1280] published a paper on a hemolytic substance in pneumococcal culture broth. No proof was presented that this substance is not the same as the hemolysin already discussed, but Sickles and Coffey, like Neill, and Cotoni and Chambrin, found that pneumococcal immune serum had a marked inhibitory effect on hemolysis, giving complete inhibition in a 1 to 1,000 dilution, whereas normal horse serum was effective only in a 1 to 400 dilution.

The belief of Cole and his associates that hemolysin is an intracellular constituent of Pneumococcus was not shared by Cowan.[287] On the contrary, this author stated positively that the principle is extracellular and arises during the growth of the organism in fluid media. Its highest titer occurred immediately after the maximal period of multiplication—phase of logarithmic growth—while the

curves of hemolytic titer and growth seemed to Cowan to denote that hemolysin production was not essentially the result of autolysis. Hemolytic power diminished in old cultures, due to oxidation, but could be restored by reducing agents. The hemolysin was not type-specific nor was it related to virulence. It filtered readily, was rapidly destroyed at 56°, was antigenic, and was produced by all but two of twenty-eight strains. The observation that the substance appears in broth cultures agrees with that of Sickles and Coffey, but it would seem that Cowan had not sufficiently ruled out the factors of autolysis to justify the conclusion that the hemolysin is extracellular in origin.

While Neill[952] found that pneumo-antihemolysin was without effect on the hemotoxins of tetanus and Welch bacilli, Todd[1412] reported that he was able, to a certain extent, to neutralize pneumococcal hemolysin and tetanolysin with the serum of horses hyperimmunized against streptococci, the action being ascribable to the content of highly active antistreptolysin in the immune serum. Todd added that the degree of neutralization was not necessarily correlated with the antistreptolytic titer and that the different hemolysins were distinguishable by quantitative serological methods. This partial antigenic overlapping of hemolysins could only be demonstrated by the use of hyperimmune serum.

Purpura Production

Somewhat analogous to the effects of the hemolysin and hemotoxin produced by Pneumococcus, but probably not a direct consequence of their action, is the purpuric condition seen in both natural and experimental pneumococcal infections. Claude[236] in 1896 was among the first to note the occurrence of purpura in an infant dying of pneumonia. The lesions tended to have black central nodes leading to ulcerative necrosis. But, more important still, the causal relation between Pneumococcus and purpura was demonstrated by the isolation by Claude of pneumococci both from the blood and from the center of the purpuric lesions. A similar case

was that described by Morse[931] in 1898, in which purpura accompanied a general pneumococcal infection in a twelve-month-old girl.

A case somewhat similar to Claude's was reported in 1914 by Rolland and Buc.[1154] The patient was an infant dying of a pneumococcal meningitis without lung involvement. Necropsy revealed intense visceral purpura, and cultures taken post mortem from the meningeal pus and from serous fluid in the purpuric lesions yielded pneumococci identical with a strain isolated from the blood during life. Reh[1123] described the case of an infant with bluish-red discoloration of the right side, left elbow, and the four extremities. Bacteriological tests showed that the infection was caused by pneumococci.

Purpura, therefore, is an occasional concomitant of pneumococcal infections, and the three cases cited are sufficient to illustrate the similarity between this change in the blood with the accompanying capillary permeability and the hemotoxic effects of Pneumococcus. The experimental facts are more pertinent to the present discussion.

In 1899, Carnot[199] observed the development of a *teinte violacée* in the nose and ears of a rabbit injected with a pneumococcal "toxin." No details of the preparation of the toxin were given but Carnot stated that it was sterile. It is probable that he employed cell-free broth cultures as did Heyrovsky.[644] The latter, by injecting culture filtrates into white mice, induced hemorrhagic dermatoses and hemorrhages of the mucous membranes and other tissues. The condition was severe and affected the internal organs as well as the skin.

Julianelle and Reimann[694] quoted Neill as having observed hemorrhagic purpura in white mice injected with an extract of Pneumococcus. The authors, by injecting extracts of both virulent and avirulent strains into white mice induced a purpuric condition manifested after four to six hours as a dark-blue discoloration of the skin of the feet, tail, ears, snout, and genitals. Unless the amount of extract was large there was no intoxication and the ani-

mals recovered. The lesions reached maximal intensity within twenty-four to forty-eight hours and disappeared in five to seven days. Extracts prepared by the Avery-Neill method were more potent than filtrates of pneumococcal cultures. Neither cultures younger than eight to sixteen hours nor those that were very old were active. Whole, unfiltered cultures rarely caused a reaction, while bile-dissolved cultures produced no purpura, although bile was shown not to inhibit this activity. The extracts induced purpura in rabbits and guinea pigs as well as in white mice. Extracts of *Staphylococcus aureus, Streptococcus viridans* and *B. coli* did not exhibit this property.

The purpura-producing principle withstands heating to 100° for ten minutes; it resists oxidation; it is filter-passing; its activity is destroyed by digestion with trypsin; and the substance can be obtained from pneumococcal extracts by full saturation with ammonium sulfate after the acetic acid-precipitable substances have been removed. The principle is common to various pneumococci and apparently bears no relation to virulence, nor is it associated with the hemotoxin of Pneumococcus, since the hemolytic activity of an extract may be destroyed without affecting the purpurigenic property.

Julianelle and Reimann concluded that the substance is a degradation product of Pneumococcus. The authors had in previous studies of experimental purpura found that excessive diminution of blood platelets, due either to direct destruction of the cells or damage to the seat of their origin, was a causative factor. In another paper, Reimann and Julianelle[1130] reported variations in the number of blood platelets in white mice injected with pneumococcal extracts. The platelets were greatly diminished after injection, the greatest decrease taking place after twenty-four hours. With a count of less than 500,000 platelets per cubic millimeter of blood, the mice usually developed purpura. A regeneration—an over-regeneration—was accomplished by the fourth to the ninth day, with a return to normal in about two weeks. The red cells were also

greatly reduced in number, but the rate of destruction and regeneration was somewhat slower than that of the platelets, while leucocytes were slightly, if at all, affected. Reimann and Julianelle emphasized the differences between the hemolysin and the purpurigenic principle in pneumococcal extracts. Both the thrombolytic and hemolytic properties were destroyed by heating *in vitro*, although such heated extracts still were able to produce purpura but not a severe anemia in mice. Extracts adsorbed with either blood platelets or erythrocytes showed a marked diminution in thrombolytic and hemolytic activity *in vitro*. Adsorbed extracts, however, caused purpura as well as severe anemia and thrombopenia in mice.

In a further study, Julianelle and Reimann[695] offered the following additional conclusions:

The purpura-producing principle of Pneumococcus is non-antigenic in the sense that it does not stimulate the formation of antibodies; white mice acquire an increased resistance to purpura as a result of repeated injections of toxic doses of the purpura substance; the serum of rabbits immunized with the purified purpura principle, with smooth and rough strains of Pneumococcus or with cell extracts, autolysates or the nucleoprotein fraction of the organism, does not confer upon white mice protection against purpura; the purpura principle does not exist preformed in the cell, but is rather an autolytic derivative, since it is formed only when pneumococci undergo autolysis, and it is not found when the autolytic ferments are inactivated; the purpura substance is associated with the proteose fraction of active pneumococcus extracts.

Pittman and Falk,[1092] still more recently, substantiated the results of Julianelle and Reimann by causing purpura in white mice with extracts of pneumococci made by alternate freezing and thawing. This extract also protected mice against an infection with pneumococci of low virulence when the extract was injected twenty-four hours prior to inoculation, but when both extract and culture were given simultaneously death ensued. The effect has some resemblances to the action of Bails' aggressin. The strange selective affinity of bacterial proteins for blood cells reminds one of the he-

magglutinative action of some vegetable proteins, especially the globulins and the proteoses of beans.

Mair,[856] differing from Julianelle and Reimann, believed that the purpura-producing principle was an intracellular substance and that the cell must be disintegrated by autolysis or other means before the active substance could be absorbed in sufficient amount to produce purpura. Another difference reported by Mair was the accelerating action of the pure bile-salts, sodium desoxycholate or sodium choleate, in releasing the purpurigenic principle. By running parallel experiments with untreated pneumococcal suspensions, some bile-treated and others containing sodium desoxycholate, Mair obtained equally good reactions. He inferred from his experiments that:

> It may be that solution of the bacterial bodies is all that is required, a preformed constituent of the cell thus being set free and rendered capable of absorption from the peritoneal cavity of the mouse. On the other hand, the delayed reactions obtained with pneumococci which had been subject only to slight autolysis, and with pneumococci dissolved in bile and immediately heated, suggest the possibility that proteolytic changes which may occur in the body of the mouse are required for the development of the substance.

Moreover, Mair found that mice varied in susceptibility to the purpura-producing substance or, perhaps, in their ability to effect proteolytic changes in the substance. By selecting for breeding mice that were sensitive to the purpura reaction, Mair discovered that this trait was hereditary, and developed a strain of mice with increased susceptibility.

That the purpura-producing principle might be associated with the carbohydrate constituents of Pneumococcus was claimed by Wadsworth and Brown[1468] and by Sickles and Shaw.[1283] The injection of the cellular carbohydrate intravenously, intraperitoneally, or subcutaneously into mice induced a purpuric condition. Notwithstanding these observations, there is a reason to believe that the effects are ascribable to small amounts of degraded protein in

the preparations of cellular carbohydrate. This substance, whatever might have been its nature, in addition to being heat-stable, was neutralized by Type I antipneumococcic serum even when the latter had been adsorbed with either the homologous soluble specific substance or the cellular carbohydrate. Sickles and Shaw reported that this purpura-producing activity of the polysaccharide was destroyed when the carbohydrate was subjected to the action of its appropriate carbohydrate-decomposing enzyme, but there is no evidence to be found in the communication that the presence of proteolytic ferments in the enzymatic extract had been excluded.

The weight of evidence favors the view that the substance responsible for the purpuric manifestations is a cleavage product of pneumococcal protein, arising in the lysis of the bacterial cell and that purpura production is not a property of any of the components of Pneumococcus as they exist in the normal bacterial cell.

Virulin, Leucocidin, and Analogous Substances

Poisoning and dissolution of red blood corpuscles and the elaboration of purpurigenic substances are not the only ways in which Pneumococcus attacks body tissues. Saline extracts of the coccus, prepared by Rosenow's method, were reported by Pittman and Falk[1092] to decrease phagocytosis of avirulent organisms, to reduce the opsonic content of serum, and only slightly to increase the virulence of a borderline culture after it had been transferred several times in the presence of the extract. The extracts failed to influence the virulence of an avirulent Pneumococcus. The principle contained in the extract, prepared by incubating dense suspensions of pneumococci in small volumes of isotonic sodium chloride solution with subsequent heating and centrifuging, the authors named "Virulin." Considering the feeble action of the substance in raising the virulence of pneumococci, the name would seem to be too specific a designation for an unidentified substance.

Another toxic principle of Pneumococcus is the "Leucocidin" described by Oram,[1032] who reported that in actively growing cul-

tures of Pneumococcus a toxin was produced that, as demonstrated
by the Neisser and Wechsberg method, destroyed leucocytes. Leu-
cocidin was present in aerobic and anaerobic cultures of both viru-
lent and avirulent strains of Types I, II, and III. It was easily
oxidized, but was not injured by exposure to 70° for one hour. The
toxin in the preparation was not destroyed by evaporation in a
vacuum at 5°, and its action could be enhanced three to four
times by the addition to the culture of laked red blood cells. Ap-
parently the same cultural conditions governed the production and
preservation of both the hemotoxin and leucocidin, although the
latter was distinguishable by its greater thermostability and its
appearance in cultures where no hemotoxin could be demonstrated.
The filtrates containing active leucocidin were not toxic for mice in
two cubic centimeter amounts injected intraperitoneally, and re-
peated doses of the filtrate gave only slight protection. Simultane-
ous injections of the extract and avirulent pneumococci did not
raise the virulence of the strain, so that the leucocidin does not
share this alleged action of the so-called virulin. Sterile superna-
tant fluids of phenolized exudates of rabbits with empyema, while
in themselves not toxic, upon repeated intravenous injection pro-
duced an antileucocidin. Normal rabbit serum did not neutralize
the pneumococcal leucocidin, whereas the majority of human se-
rums tested possessed neutralizing properties.

One more morbid effect elicited by pneumococcal extracts or
autolysates deserves attention and that is the necrotizing principle
reported by Parker.[1059-60] This principle is a filtrable substance, is
extremely thermolabile, is sensitive to oxidation, and can be sepa-
rated from hemotoxin by adsorption with red blood cells, since the
necrosis-producing principle remains unaffected after removal of
the hemotoxin. The necrotizing substance or substances obtained
from both Types I and II pneumococci are neutralized by Type I
antipneumococcic serum, and hence the necrotizing substance is
not type-specific.

The virulin, leucocidin, and, to coin a name, the necrotin have

been described in some detail as representing one of the recent trends in the investigation of Pneumococcus and to draw attention to still unstudied possibilities of this remarkable cell and its properties.

So-Called Toxins

The severe intoxication that so frequently accompanies pneumococcal disease naturally led early investigators to search for a soluble toxin. Profoundly impressed, as they must have been, by the discoveries by von Behring and Kitasato and by Roux of diphtheria and tetanus toxins and of the dramatic efficacy of the corresponding antitoxins, there came a vision of a new and similar agent for the treatment of pneumonia.

Foà,[458] in 1890, thought that he had found a poison in pneumococcal cultures. He isolated the supposed principle by precipitation with ammonium sulfate, dialysis, and subsequent concentration. The extract thus prepared produced marked biological changes in rabbits, but failed to kill the animals. Repeated injections raised the resistance of the test animals to later inoculation with a virulent culture, but it seems safe to infer that Foà was merely dealing with the deleterious degradation or lytic products of Pneumococcus. In the next year, Bonome[137] succeeded in preparing filtrates that were lethal for rabbits. The toxicity was directly related to the virulence of the culture, and the more poisonous extracts, after repeated doses given subcutaneously, intravenously, and intraperitoneally, rendered rabbits resistant to infection. But the subsequent effects were neither toxic nor antitoxic in the true sense of the terms. Issaeff,[673] at the Pasteur Institute, reported similar experiments with comparable results. The serum of rabbits treated with broth cultures of low toxicity possessed therapeutic but no antitoxic power. Issaeff, therefore, had not demonstrated the existence of a toxin. Bunzl-Federn[186] also tested in a similar way heated broth cultures but found that their toxicity and antigenicity were feeble. Cole[250] was able to elicit more severe

and fatal toxic or poisonous effects with saline extracts and bile solutions of pneumococci. The intravenous injection of the latter materials into guinea pigs initiated a train of symptoms terminating in a type of death resembling that seen in acute anaphylaxis.

In the same year, Rosenow[1166] published a series of four papers, in which he described observations similar to those of Cole, accompanied by an explanation of the observed phenomena. In the first article Rosenow reported that a single injection of saline autolysates of Pneumococcus, Pneumococcus-leucocyte mixtures, pneumococcal exudates, and mixtures of the organism with normal or immune serum, all produced identical symptoms in normal guinea pigs, and that the symptoms were those of acute anaphylaxis. The serum of animals sensitized to Pneumococcus when allowed to act on the organisms produced this toxic substance more rapidly than did normal serum, and this toxin production as measured by the polariscope was accompanied by a more rapid proteolysis. Morphine, ether, urethan, atropin, and adrenalin protected normal guinea pigs against the poisonous action of the preparations just as they protected sensitive pigs on re-injection. Rosenow's conclusions are significant:

> The behavior of normal and sensitized guinea pigs toward unautolyzed extracts of pneumococci, which are non-toxic to the former and very toxic to the latter, toward partially autolyzed extracts which are very toxic to the former, and slightly or not at all to the latter, and toward more completely autolyzed extracts, which are non-toxic to both, speaks strongly in favor of the view of a rapid parenteral digestion into toxic cleavage products in sensitized animals.

He added that the cleavage products formed *in vivo* were identical with the toxic substances obtained *in vitro*, and that the appearance and disappearance of toxicity seemed to be definitely related to proteolysis. Rosenow also reported that a single intravenous injection of non-fatal doses of extracts before they had become toxic, or while highly toxic, and especially after the toxic stage had been passed, or of autolyzed pneumococci, rendered guinea

pigs insusceptible to subsequent injections of toxic pneumococcal autolysates. Although the duration of this protection was not mentioned, the statements point to a refractory state or temporary tolerance so characteristic of degraded proteins and not to an immune condition.

In a second communication, Rosenow[1167] described other characters and properties of the poisonous substance. It was destroyed when the clear autolysate was heated for twenty minutes at 60°, as well as by weak solutions of hydrochloric acid; it was adsorbed by animal charcoal from which it could be recovered by ether. From its chemical behavior the author decided that the toxin was probably a base containing amino groups. He concluded by saying, "Indications have been obtained showing that during pneumococcus infections toxic substances are produced which do not call forth any immunizing response."

In a third paper, Rosenow[1168] ascribed the production of the toxic material to the action of a proteolytic enzyme present in extracts and autolysates of the pneumococcal cell, and in a concluding report[1169] described the action of various pneumococcal products on dogs. The symptoms and lesions were strikingly like those observed in immediate canine anaphylactic shock. The hemorrhages, especially those in the intestines, the effect on the respiration, the extreme degree of cyanosis, the delayed coagulation of the blood, and the presence of carbon dioxide in the stomach indicated to Rosenow that the chief effects of the toxic substances were due to an interference with normal oxidative processes. The action of the autolysates closely resembled that of protein cleavage products and, according to Rosenow's view, it made no essential difference whether the poisonous substances were formed *in vitro*, in the consolidated lung in man, or at once in sensitized dogs, since they were all of the same general nature and their effects differed only in degree and not in kind.

The anaphylactoid shock seen after the intravenous injection of pneumococcal extracts into guinea pigs has also been reported by

Weiss,[1509] who obtained an analogous reaction in rabbits. He saw no parallelism between the toxicity and hemolytic power of the extracts, nor any neutralizing action by antipneumococcic serum. Weiss, like Rosenow, found a similar toxic principle in lung exudates of patients suffering from acute lobar pneumonia. Clough's[241] paper (1915) contained similar observations. In the same year, Boehncke and Mouriz-Riesgo[134] failed to isolate the toxic substance from young cultures or from sodium taurocholate solutions of virulent strains. Contrary to earlier reports, these two authors found that the serum of rabbits treated with these preparations displayed antitoxic action but did not affect the infective process. There followed a paper by Weiss[1509] on "Pneumotoxin," in which he repeated Cole's observations on the anaphylactoid reaction caused by sodium choleate solutions of living, virulent, washed pneumococci. The description of the properties of the preparations agrees with previous accounts of the characters of pneumococcal hemotoxin.

Chesney and Hodges[221] departed from the idea that the intoxication in pneumonia was due to a toxin, believing that it was more reasonable to associate the phenomenon with the growth of the organisms, rather than with their death and dissolution. The authors' attempts to detect toxic substances in fluid cultures of pneumococci during the period of active growth proved entirely negative.

The demonstration of a toxin was claimed by Olson[1029] who experimented with sterile sodium ricinoleate solutions of pneumococci. He also claimed that the intraperitoneal injection of such preparations produced cutaneous reactions in animals and caused clinical and pathological symptoms of pneumonia. An antiserum against this substance appeared to prevent to a high degree both pulmonary and cutaneous reactions. Olson stated without qualifications that a toxin had been demonstrated, that the action of the immune serum was antitoxic, and that "indications are that the toxin may be of value in the production of active immunity to

pneumococcus infections." In the light of later events this claim and hope would seem to be premature. Larson,[787] with pneumococcal filtrates supplied by Olson, found the same congestion of the lungs in white mice injected intraperitoneally. Of skin tests performed with the filtrates, those on pneumonia convalescents were of possible significance in being uniformly negative. Larson voiced the same conviction that the filtrates contained a soluble toxin.

In a second paper, Olson,[1030] after describing the lesions produced by pneumococcal filtrates in mice, went so far as to say that the toxin was an exotoxin and not an autolytic product or endotoxin, and gave as his reason that eight-hour culture filtrates possessed marked toxicity but contained little hemotoxin. The toxic principle was relatively thermostable, but was completely destroyed by boiling for one hour. While unpreserved lots lost activity, 0.3 per cent cresol served to maintain the toxicity for several months regardless of the temperature. Active immunization of mice by serial injections of the toxin resulted in protection against 1,000 to 10,000 fatal doses of pneumococci, while the specificity of the immunity induced bore no relation to the serological type of the organism from which the so-called toxin was obtained. The stability of the toxic principle in the presence of heat, the stabilizing action of cresol, and the lack of specificity are, as we know, characters not shared by true exotoxins.

The report of Parker and Pappenheimer[1063] takes us back to the toxic action of autolysates. Solutions were prepared by allowing a suspension of pneumococci, sealed in tubes under vaseline, to stand in the dark at 22° to 24° for two to five days and then in the ice-box. The autolysates were centrifuged in the cold, the seals opened, and the clear supernatant fluid passed through a well-iced Berkefeld filter. The filtrate injected intratracheally in a dose of 0.2 cubic centimeters was highly toxic for guinea pigs, death taking place either within a few hours or within three days. In animals succumbing early, there was intense hemorrhagic edema of the lungs with beginning inflammatory reaction; in animals surviving

for eighteen hours or longer, extensive areas of pneumonia were found. As a control, the authors made intratracheal injections of living, virulent pneumococci which were followed by a transient, slight lesion and recovery, or later by death from septicemia without pneumonic lesions. The addition, however, of a sublethal dose of toxic autolysate to the living pneumococci altered the reaction of the animal so that an extensive pneumonia developed associated with unrestrained multiplication of the organism. This synergistic property was far more pronounced than that of the virulin of Pittman and Falk and was more like that of an aggressin.

Yamamoto (1929)[1557] listed unheated pneumococcal filtrate, heated filtrate, and "standard pneumococcus vaccine" (?) according to toxicity in a ratio of 1 to 2 to 6, but none of the three was notably toxic, not less than 6.0 cubic centimeters of the last-named preparation being required to kill a rabbit.

In 1929, Parker[1060] returned to a study of the necrotizing substance of Pneumococcus in comparison with the lung-toxic principles in autolysates. She found both substances to be sensitive to heat and to oxidation, and both were neutralized by the same anti-autolysate serums. The lung-toxic principle, however, was absorbed or inactivated by red blood cells, whereas the necrotizing principle was not. In the discussion, Parker wrote:

> Since pneumococcus hemotoxin is present in the anaerobic autolysate and is also absorbed by red cells, it seemed possible that it was this substance in the autolysates which caused the diffuse lung lesions and death of guinea pigs. However, it was found that the intratracheal injection of pneumococcus hemotoxin prepared by the method of Avery and Neill only occasionally produced the characteristic reaction caused by the intratracheal injection of the anaerobic autolysates. From these experiments we believe, therefore, that the necrotizing and lung-toxic principles, and probably the pneumococcus hemotoxin also, are all separate entities in the anaerobically produced autolysates described.

Pittman and Southwick[1093] were also attracted by this problem. In their experiments, the injection into mice of extracts produced

by repeatedly freezing and thawing pneumococci was followed by marked hemorrhagic lesions on all or a part of the external surface that was free from hair, and at necropsy hemorrhagic areas could be found in practically every tissue of the mouse. The adjuvant action of the extract was seen when mice, dying after injection with extract followed several hours later by culture inoculation, developed more marked hemorrhagic lesions than any other group of mice studied. Fresh filtrates of virulent pneumococci produced slight pathological reactions in mice but, when the filtrates were injected with a culture of low virulence, 71 per cent of the mice at necropsy showed a fibrino-purulent pleuritis. The results might be interpreted as pointing to the production of an aggressin-like substance in the early stages of pneumococcal growth with the development of a more violent poison as growth continues and the cells undergo autolysis.

Jamieson and Powell[676] also described a skin-reacting substance in pneumococci. To them its properties were comparable to those of the toxins of some streptococci. The alleged success in developing a neutralizing antiserum led the authors to believe that the present type of antibacterial immune serum would be more valuable if it contained these antiskin-toxic elements. Sabin (1931),[1203] investigating the part anaerobic autolysates of Pneumococcus play in the course of natural infection, came to conclusions diametrically opposed to those of Parker and the other students sharing her ideas. Sabin wrote: "The only conclusion that may be drawn, however, is that the anaerobically produced toxins probably do not play any part in the causation of death of mice infected with very large doses of pneumococci." The failure of "antipneumotoxic" serum to modify the course of pneumococcal infection led Sabin to conclude that: "It seems fair to assume that the anaerobically produced toxins are probably products primarily of the enzymatic changes occurring in *in vitro* autolysis, and play no part in natural infections." The idea of producing an immune serum capable of neutralizing the poisonous principles of Pneumo-

coccus still persists. Coca,[246] after continuing the investigation of
the toxic principle in Pneumococcus, announced additional data in
1936. When the pneumococcal cultures were grown in the presence
of oxygen a toxic substance was formed but it was devoid of anti-
genic properties. When, however, carbon dioxide was supplied to
the growing cultures, the sterile filtrates were not only toxic but
antigenic. According to Coca, the pyrogenic toxin seemed to be
type-specific and was not the same as the type-specific polysac-
charide. Evidence was presented purporting to show that the in-
jection of the toxic filtrates into the human body or pneumococcal
disease in human subjects provoked neutralizing substances in the
serum for filtrates of the homologous type, which the author be-
lieved were specific antitoxins.

A careful reading of original papers dealing with the subject of
toxin production by Pneumococcus fails to bring conviction that
the substances or extracts described should be looked upon as
true soluble toxins. The principle involved in their formation or
preparation, that is, disintegration of the pneumococcal cell by
self-proteolysis, bile solution, or freezing and thawing; the nature
of the local and systemic reactions the materials evoke; the acute
anaphylactoid death produced in guinea pigs; and the feeble anti-
genic powers, all argue for the classification of the so-called toxins
with the proteins of smaller molecular size formed in the hydrolysis
of whole proteins. One has only to recall the work of Vaughan[1447]
and of others on the effects of the cleavage products of bacterial
and of pure proteins, and the many studies on proteoses and pep-
tones, to be impressed by the similarity between the alleged pneu-
mococcal toxins arising from the autodigestion of the somatic pro-
tein of Pneumococcus and protein poisons. The slight antigenic
power of the pneumococcal derivatives and their seeming lack of
specificity is easily explained when one remembers that the hy-
drolysis of proteins is not always an orderly, step-by-step process.
Along with the formation of proteoses and peptones, or polypep-
tids and amino acids there may be traces of proteins of larger

molecular size, even to whole unsplit proteins, remaining in digestion mixtures. Traces of intact proteins acting as antigens may be responsible for the weak neutralizing power of the serum of animals treated with extracts, filtrates, and autolysates of Pneumococcus or, what is more probable, such feeble and transient resistance as these antigens induce is merely the tolerance established by degraded proteins and not immunity.

While it is possible that a true toxin may be a constituent of the pneumococcal cell or a product of its metabolic processes, until more convincing evidence is presented, it would seem reasonable to look upon these so-called "toxic" effects as being referable to the action of bacterial protein poisons.

Summary

It may assist the reader to gain a clearer conception of the relation of the biochemical phenomena manifested by this bacterial cell to the morbid processes induced by the living organism to select from the mass of data the more significant discoveries and to weave them together into a summary. Pneumococcus is a fragile body and contains within itself enzymatic forces that lead to its disruption and disintegration, rob the substrate in which it lives of nutrient substances, and from these substances evolve chemical agents that arrest further growth, cause the death of the organism, and affect the cells of the animal body into which the microbe may find its way.

Intracellular proteases break down the proteins of the cell and of the substrate upon which the cell feeds into smaller fragments, which in turn are still further reduced in size by the same ferment or possibly by a peptidase. This proteolysis reduces the food supply in the medium, and the products of the digestive action may induce purpura and cause other more or less violent toxic effects in the animal host. The poisonous principles, as far as can be learned, are not true toxins, but resemble degraded proteins in their physiological action. Pneumococcus is also endowed with saccharolytic

enzymes, capable of attacking starch, inulin, and glycogen; invert-
ases that convert complex saccharides into simpler sugars, and
other ferments that split these sugars into acids, while the acid so
formed arrests further proliferation of the bacterial cell. Intra-
cellular lipids are converted into fatty acids by pneumococcal
lipases and therefore the self-destruction of the cell may be com-
plete. Furthermore, the ingredients of the medium in which the or-
ganisms grow are largely replaced by a new order of constituents.
The action of all these enzymes may be reversible and by their ac-
tion or that of some similar agents Pneumococcus is able to build
protein, lipids, and somatic and capsular polysaccharides from
substances present in the substrate.

In the operation of these vital processes, oxygen plays an im-
portant part. The element is essential to the hydrolysis and syn-
thesis of protein, lipid, and sugar but it may act in a variety of
ways. Some of the products of oxidation are inimical to the nor-
mal functioning of the bacterial cell and some affect changes in the
respiratory mechanism of the blood. Pneumococcus utilizes oxy-
gen to form peroxide that is toxic to the cell. Peroxide destroys the
labile constituents of the pneumococcal cell, which with the easily
oxidizable intracellular substance continue to form an oxidizing
substance responsible for the conversion of oxyhemoglobin into
methemoglobin.

Pneumococcus, therefore, by the action of its intrinsic enzymes
may destroy itself and by the operation of its oxidation-reduction
system and its extracellular derivatives or products may, through
the dissolution of red cells, the conversion of oxyhemoglobin into
methemoglobin, and the lowering of oxygen capacity of the blood,
wreak serious damage to the tissues and pervert the normal physio-
logical functions of the body of the infected host.

CLASSIFICATION OF PNEUMOCOCCI

*The separation of the members of the species into specific types
by immunological reactions, with a description of methods for the
determination of these serologically specific types.*

B Y tinctorial and cultural methods it is a relatively simple task
to differentiate pneumococci from other bacterial species but
it is impossible by these methods, except in the case of Type III
organisms, to distinguish pneumococci of one serological type
from those of another.

The lance-shaped form, with the presence or development of a
capsule, and the appearance of the colonies on blood agar, with
their zone of greenish discoloration, are presumptive signs of the
identity of these cocci. Solubility in bile, fermentation of inulin,
marked pathogenicity for mice and rabbits, and sensitiveness to
optochin are all characters which make possible a positive identifi-
cation of the species. The possession of these characters has defi-
nitely placed the former bile-soluble *Streptococcus mucosus* among
the pneumococci, and its mucoid growth, thick capsule, and greater
virulence serve to separate it from pneumococci of some of the
other types. However, it is by their serum reactions that the sepa-
ration of pneumococci into definite immunological types has been
accomplished.

Serological Classification: 1898–1932

It was undoubtedly Bezancon and Griffon[108-10] who, in 1897,
were the first to report a discovery that later was to establish dif-
ferences in the serological behavior of pneumococci as a basis for
an invaluable aid in all phases of research on Pneumococcus. While
Metchnikoff[394] and later Mosny[933] had previously observed the ag-
glutinative action of antipneumococcic serum, it was Bezançon and

Griffon who announced that from the standpoint of agglutination there existed several races of pneumococci that behaved serologically as though they were different microbes.

At about the same time, Mennes (1897),[893] with a highly potent serum prepared by the immunization of goats and horses with a single strain of Pneumococcus, was able to demonstrate wide agglutinative coverage for other strains irrespective of their origin, whether from cases of pneumonia or from normal mouths. Kindborg* took the view that pneumococcal antibodies, whether agglutinins or protective antibodies, were strictly strain-specific. Two years later, Eyre and Washbourn[876] confirmed Bezançon and Griffon's discovery by means of another immunological reaction. When they tested the protective action of a specific immune serum against five strains of pneumococci, they obtained excellent protection in mice against four of the strains, but none against the fifth. From the experience, the authors concluded, "There exist varieties of pneumococci which at present are only distinguished by the action of antipneumococcic serum."

In the next year, Bezançon and Griffon,[113] continuing their study of the agglutination reaction, noted that the serum of a pneumonia patient was most active against the particular strain causing the infection. Among the experimental data was included the description of a strain that must have been a Type III pneumococcus, since its unusually large capsule and the white, viscous peritoneal exudate it caused in white mice were characteristic of that type. The authors said that the strain was also distinctive in its serum reactions, but unfortunately did not specify in what respect. In a separate paper a year later (1901), Eyre[371] reported that agglutinability of pneumococci depended upon an optimal reaction of the medium in which the organisms were grown. This observation was partly confirmed by Yoshioka[1564] in 1923.

The contribution of Collins (1905)[270] to the serological differentiation of pneumococci seems to have been overlooked by some

* Quoted by Neufeld and Schnitzer.

authors and reviewers. She prepared agglutinative serum by im-
munizing rabbits with repeated doses, first of killed, then of living,
and finally of killed broth cultures of pneumococci. The strain used
as an antigen must have belonged to a type other than I or II,
since the serum agglutinated only a few of the seventy strains
tested. Collins' results may be summarized as follows: Pneumo-
cocci by reason of their agglutinating properties exhibit a tend-
ency to separate into numerous groups; *Pneumococcus mucosus*
forms a distinct and consistent race, and the resistance of the ag-
glutinins produced by it to absorption by streptococci indicates a
nearer relation to Pneumococcus than to Streptococcus. There
was considerable uniformity of reaction of the various strains in
low dilutions, but this uniformity was not continued as the animal
became more highly immunized, and it was not possible for Collins
to establish a definite relationship between the agglutination reac-
tion and the other characters of Pneumococcus except in the case
of *Pneumococcus mucosus*.

In 1906, Eyre, Leathem, and Washbourn[372] suggested the sepa-
ration of pneumococci into two groups according to the reaction
of rabbit tissue to infection, that is, a fibrinous type and a cellu-
lar type, but this idea apparently was never pursued.

It remained for Neufeld and Haendel[991] in 1910 to demonstrate
the full significance and the differential value of these phenomena.
With a collection of pneumococcal strains isolated from a series of
pneumonia patients the authors obtained, by the immunization of
rabbits, asses, and horses, monovalent serums of high potency.
With a culture isolated in 1909 a serum was prepared that pro-
tected mice against the majority of the other strains, and this
strain was called "Pneumococcus I." All the pneumococci against
which the serum protected mice were classed as "typical," and all
others as "atypical." Among the latter was one strain, "Franz,"
that was later found to be Type II. The "typical" antiserum had
no effect on the "Franz" strain, and a "Franz" antiserum failed
to protect mice against the Type I culture. Neufeld and Haendel

found that the results obtained by protection experiments agreed with those of the agglutination reaction. On the basis of these discoveries Neufeld and his colleague were the first to recommend that serums should be developed for all types, since it was thought that there were probably many types and that a given serum always exhibited type-specificity. For the serum treatment of pneumonia the authors suggested that the agglutination test be employed to determine the type of the infecting organism before administering the specific serum.

The next impetus to serological classification of pneumococci came in 1913 from the work of Dochez and Gillespie.[322] These authors, by the methods of protection and agglutination, divided the species into four groups. Groups I and II included over 60 per cent of the strains tested, Group III consisted of organisms of the *Pneumococcus mucosus* type, and Group IV was a heterogeneous collection of strains that fell into none of the first three divisions and that reacted only with strictly homologous, that is, individual, strain-specific serum. When fourteen different cultures were tested against eleven serums for these heterologous strains, no cross-protection was seen, except in one instance.*

Dochez and Gillespie's Type I Pneumococcus corresponded to the strain originally isolated by Neufeld and Haendel in 1909 and sent by the latter authors to the Rockefeller Institute. The American Type II was identical with Neufeld and Haendel's "Franz" culture, which was a representative of the most commonly occurring of the atypical strains. Group IV of the American authors Neufeld preferred to call Group X, and this designation is often found in the German literature.

The typical and atypical strains of Neufeld and Haendel had, therefore, now been sorted into three definite and specific types and a heterogeneous group of wide immunological diversity.

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* Wirth,[1524] in a study (1927) of pneumococci from infections of the ears, sinuses, and throat, found strains of the *mucosus* type which, irrespective of the presence or absence of slimy growth on blood agar, agreed in their agglutinative characteristics with those of the American Type III. Wirth recommended that the name *Streptococcus mucosus* therefore be abandoned.

Lister (1913),[815] studying pneumococci recovered from native laborers in South Africa, was struck by the comparative rarity with which recently isolated pneumococci were opsonized even by serum of pneumonia patients. He succeeded however in obtaining opsonic preparations which showed massive agglutination and marked phagocytosis. Tests on his first four cases suggested the "existence of groups of pneumococci, having distinct serological reactions," and the "presence of specific agglutinins in 'critical' sera from pneumonic patients." Serum from a Group I case failed to react with cultures of Group II, III, or IV pneumococci, and serum for Groups II, III, and IV failed to react with a Group I culture, but any of the Group II, III, and IV serums reacted typically with any of the cultures of Groups II, III, and IV. The numerals are Lister's and bear no relation to the designations used by Dochez and Gillespie. It was not until the completion of his work that Lister learnt of the earlier classification. He immediately compared his groups with those of the American authors, and divided his twenty cultures into five groups, A, B, C, D, and E, of which Group A could not be identified with any of the American types, while Group C corresponded to Type I, and Group B to Type II. Lister's E group was later found to be the same as Type III.

A year later, Lister[816] was able to clarify the apparent confusion existing between his classification and that of Dochez and Gillespie. He sent to the American workers dried spleens of mice infected with cultures representing his groups and received from them in return samples of Type I, II, and III serums. This mutual investigation showed that Lister's C and B groups corresponded respectively with the American Types I and II. Since Lister had no E cultures at the time, he could not correlate it with any of the American groups. At the Hospital of the Rockefeller Institute, however, the identification of the E strain as Pneumococcus III was made. In regard to Dochez and Gillespie's Group IV, Lister stated that it was at this point that his classification departed

widely from that of the workers at the Rockefeller Institute. In the
Transvaal there were a number of additional groups, and Lister's
A was of great importance, since its members were more prevalent
than those of any of the groups C, B, or E, and caused a higher
case fatality. His unclassifiable groups D, F, G, and X Lister con-
sidered of less importance, because of the fluctuation in their
prevalence and low case mortality. The pneumococci in these
groups showed strain-specificity with no cross-reactions in agglu-
tination. Lister's A and D Group cultures were placed by Dochez
and Gillespie in Group IV.

Dochez and Avery,[319] in their 1915 paper, explained that pneu-
mococci of Groups I, II, and III were found principally in asso-
ciation with disease and were distinctly parasitic in type, while
Group IV comprised a heterogeneous series of strains, not related
antigenically, which caused a minority of cases of pneumonia, and
from which the pneumococci occurring in the normal mouth were
indistinguishable. Then Avery,[33] by means of agglutination and
protection tests, further divided Type (originally Group) II
pneumococci into three subgroups, designated by him as "IIA,"
"IIB," and "IIX." Protection tests showed that organisms of any
subgroup were not protected against by the serum of other sub-
groups, nor did the strains absorb from Type II antipneumococcic
serum the specific immune bodies of the other subgroups. Avery
stated that the organisms of the three subgroups were biologically
related to Type II Pneumococcus, that organisms of subgroups
IIA and IIB were characterized by immunity reactions identical
within the respective subgroup, but that subgroup IIX consisted
of heterogeneous strains which did not cross with other strains or
with Types IIA or IIB.

In 1916, Olmstead[1027] after testing the agglutinative reaction of
over two hundred strains of pneumococci isolated from normal and
infected human beings against fifteen serums, including Type I and
Type II serums, against which all those strains failed to react,
confirmed the validity of the classification of pneumococci into

Types I, II, and III and then divided the members of Group IV into at least twelve groups, some of which contained subgroups. In a subsequent report, Olmstead[1028] suggested that some members of these groups served as connecting links between Type II and Group IV, and, because of a closer relationship with the latter, should be classed as Pneumococcus IV rather than as IIX. The same proposal was made by Clough,[238] who after identifying strains of Avery's subgroups IIA and IIB among 121 cultures isolated from cases of lobar pneumonia, suggested that, since members of these two subgroups possessed relatively low virulence for animals and had been recovered from the mouths of normal persons, the strains would be found epidemiologically to resemble Group IV organisms more closely than those of Types I or II.

Nicolle, Jouan, and Debains[1011] did not accept Group IV and the American classification, claiming that a large number of pneumococci studied by them were not agglutinated by their own or American serums, except when treated with dilute hydrochloric acid according to the method of Porges. The authors recommended that the conception of Type IV—a purely negative type—ought then to be abandoned henceforth.

Nicolle with Debains[1009] again studied by the agglutination reaction a large number of pneumococci from varied sources. They used the American classification and their results showed that the strains, as studied, varied in agglutinability from a complete absence of this property to spontaneous clumping in normal horse serum.

In a review of various immunological reactions, Nicolle and Debains (1920)[1010] concluded that races of Pneumococcus—"antigenic races"—could not be determined by agglutination alone. Their work is, in one respect, reminiscent of Gillespie's[516] data on the acid agglutination of pneumococci. Tested by this method, strains belonging to Types I and II had, as a rule, narrow zones of agglutination. Other pneumococci had broad zones, or in a few cases, narrow zones not coincident with those occupied by organ-

isms of the fixed types. The acid-agglutination of the majority of pneumococci of Types I and II was extremely susceptible to the inhibiting action of salts, but this was not true of other pneumococci. These influences, with the variations they cause, while of a certain philosophic interest, have little bearing on the practical application of agglutination to the classification of members of this bacterial species.

Following the lead developed by Avery, Stillman[1327] further subdivided Type II strains into twelve distinct groups. These were designated as IIa to IIm. Groups IIb–c–f–m originated in human mouths. Groups IIa and IIh were encountered largely in connection with disease, while the general fatality of acute lobar pneumonia due to these atypical Type II pneumococci was fairly high. Stillman's tables, showing the percentage of incidence and of mortality of strains of these subgroups, offer an interesting comparison with those given later by Cooper and her associates. They serve to emphasize the close relation which exists between some members of the Type II and those of the new types formerly included in Group IV.

In conformity with Stillman's and Olmstead's investigations, Christensen proved that Type IVm of Stillman also constituted a heterogeneous group which could be further divided into subordinate groups. Christensen[228-9] carried out comparative tests by means of agglutination, agglutinin-absorption, and complement fixation and gave preference to the simple agglutination technique as being the most convenient and efficient method for serological differentiation.

The splitting of Type II into separate groups was apparently unknown to Hintze,[646] who found anomalies in the American Type II and was not convinced that it was a clear-cut group. He, among others, also classified *Streptococcus mucosus* as Type III Pneumococcus, although strains of this organism were encountered that gave atypical reactions.

Yu,[1567] studying fifty-one strains by the agglutination method,

found at least three subgroups in Type II. Sugg, Gaspari, Flem-
ing, and Neill[1354] in 1928 described a peculiar strain which, while
possessing a partial antigenic relationship to typical Type III
Pneumococcus, had distinct immunological properties of its own.
In a later study of this same strain, Harris, Sugg, and Neill[595] as-
certained that in rabbits this culture, related to but not identical
with Type III, evoked better antibody (agglutinin) response than
did Type III organisms, but in mice the Type III strain gave rise
to a higher degree of protection against the homologous organism.

Other serological variants were found by Clough (1919).[239] Her
nine strains were agglutinated by Type I, II, and III antiserums.
From the I and II serums, by absorption, two strains removed the
agglutinins and tropins for the homologous cultures only and not
for typical I or II organisms or for other atypical pneumococci.
Absorption of the serums with homologous cultures removed ag-
glutinins and tropins for all the atypical strains. The phagocytic
and agglutinative reactions of the atypical organisms in monova-
lent serums indicated that, in general, the strains were serologi-
cally distinct, although in a few cases they exhibited some interre-
lation. The special atypical monovalent serums showed no activity
with Type I, II, or atypical Type II pneumococci.

Another atypical strain of the heterogeneous Group IV was de-
scribed by Pockels,[1101] who thought that its growth was sufficiently
individual to warrant his calling the strain *Pneumococcus planus*.
This organism has not been further identified. Gordon,[543] testing
fifteen strains of Group IV by cross-agglutination and agglutinin-
absorption, sorted them into three groups and eight separate het-
erologous strains.*

In England, Armstrong[19] was able to identify subgroups among
Type II pneumococci which corresponded to Avery's IIA and IIB
and, by means of agglutinin-absorption, divided Types I and III
into subordinate groups of limited specificity within the main type

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* For a review of serological classification as it stood in the year 1922, and
for a very readable discussion of the theory of antigens and antibodies, the
reader is referred to Eastwood's[343-4] two papers published in that year.

to which they belonged. Armstrong regretted the use of the American designation Type IV for the unclassified strains as being too narrow. Yu[1567] found Types I and III to be uniform and definite.

In 1921, Cooper, with Mishulow and Blane,[273] published the first of a series of studies which were to bring a new order out of the confusion regarding the proper serological classification of Group IV pneumococci, and to establish many of the atypical subgroups as separate and definite types. By the agglutination method, checked by absorption tests when cross-agglutination occurred, the authors placed their fifty-three cultures in six small groups with thirteen unrelated strains—a total of nineteen types.

In the next few years there were additional reports on the serological classification of pneumococci, but only a few were of major importance. In 1921, Griffith[558] compared strains of his own isolation with standard type strains sent him from America by Flexner. To a 1 to 10 dilution of the monovalent type rabbit serums he had prepared was added an equal amount of the supernatant fluid from centrifuged peritoneal washings from mice inoculated with pneumococcal strains. It was found that the agglutination test was sufficient for the identification of the first three specific types and the author considered that the method of absorption of agglutinins was unnecessary. Group IV was separated into six types, including the American Types IIA and IIB, but these did not complete his classification. Griffith, by the way, like others, noted that agglutinating serum from horses was less selective than that of rabbits.

Yoshioka (1922 and 1923),[1560-1] using strains of the American Types I, II, and III from the Hospital of the Rockefeller Institute, and a Type I strain of German origin, was not always able to obtain type-specific protection in mice actively immunized with these cultures. The serum of immunized rabbits was type-specific in protection experiments, but fresh normal serum in fairly high dilutions sometimes also agglutinated heterologous types. Yoshioka

attributed these variations in normal specificity, such as marked decrease in agglutinability with homologous serum and the appearance of agglutinability with heterologous serum, to serological modification brought about by growth of the organisms on unfavorable media.

Takami (1925)[1373, 1375] had even less success in efforts to classify Japanese strains according to the American scheme, and said that all such attempts had completely failed in Japan. It seems highly improbable that specific types of pneumococci are not to be found in that country. The various modifications which the American strains underwent on artificial media may explain the unusual results. Takami offered this fact as an explanation when he concluded that "for serological grouping of pneumococci one must work only with strains which have become virulent through animal passage, or strains which have been cultivated directly from the human body and have not undergone variation on artificial mediums." Megrail and Ecker,[888] on the contrary, presented definite evidence that Pneumococcus possesses a type stability under conditions in which typhoid bacilli and other organisms show variability in agglutination. The reliability of the methods for the serological typing of pneumococci is further supported by the results of Christensen[229] and of Griffith,[558] who demonstrated the complete specificity of the mouse-protection test.

In 1929, Cooper, with Edwards and Rosenstein,[272] tested 120 strains of pneumococci isolated from cases of lobar pneumonia that either did not agglutinate or else reacted atypically with diagnostic antiserums for Type I, II, and III pneumococci. These authors included Avery's IIA and IIB strains, representatives of various types from Pittsburgh, and the atypical III strain described by Sugg, Gaspari, Fleming, and Neill.[1354] Cooper and her associates prepared monovalent serums from rabbits and horses, and with serum divided the 120 strains into ten groups containing four or more strains each, and a miscellaneous group comprising

strains differing from the others, which at that time could not be further subdivided. These ten groups were designated as Types **IV** to and including **XIII**.

Finding that therapeutic antiserums for Types I, II, and III had little protective power against Types IV to XIII, the authors prepared from rabbits monovalent antiserums of high agglutinative and protective titer for each type. The Cooper Type IV included an "Antitoxin" strain from the Lilly Laboratories, Robinson's Group IVB strain, and one that corresponded to Griffith's 10. These strains were highly virulent for mice, and of the nineteen human cases of lobar pneumonia from which pneumococci were isolated, sixteen were rated as severe. Type V included Avery's IIA and Robinson's IVE strains. All showed a tendency to hemolyze red cells in blood broth, and had a high initial virulence for mice, which however was rapidly lost. Type VI strains were usually less virulent for mice and their hemolytic action less marked than that of Type V, but more pronounced than that of the majority of other strains. Type VI corresponded to the largest group separated by Griffith from Group IV and found by him to be second in prevalence to Types I and II. The Type VI strains had such a low virulence for mice that they were not suitable for protection tests, nor could their virulence be sufficiently increased for this purpose. Type VIII included Robinson's Group IVA organism and the atypical Type III strain described by Sugg and his associates. These strains agglutinated with Type III serum to such a marked degree that they might easily have been identified as belonging to this original type. All Type IX cultures showed low virulence for mice. The Type X strains possessed slight or moderate virulence. The four strains designated as Type XI exhibited moderate hemolytic action and were of average to high virulence for mice, which however was quickly lost. The Type XII strains were moderately virulent for mice. The virulence of the Type XIII strains was similar to that of Type XI.

In 1932, Cooper, with the collaborators Rosenstein, Walter, and

Peizer,[274] expanded the classification to include twenty-nine types in addition to the first three original types of Dochez and Gillespie, making a total of thirty-two specific types. To show how the new types compared with atypical strains described by other authors, the following list taken from the paper by Cooper and her associates may be repeated here:

Type IV—Pneumococcus 10 (Griffith), Group IVB (Robinson)
Type V—Sub IIA (Avery), Group IVE (Robinson)
Type VI—Sub IIB (Avery)
Type VIII—Group IVA (Robinson), atypical III (Sugg, Gaspari, Fleming, and Neill)
Type XV—Pneumococcus 98 (Griffith)
Type XXI—Pneumococcus 160 (Griffith)
Type XXII—Pneumococcus 41 (Griffith)

The majority of the thirty-two types showed only slight cross-reactions; Types II and V; III and VIII; VII and XVIII; and XV and XXX being exceptions. Only a small percentage of strains isolated in New York City could not be serologically identified by Cooper and her co-workers.

In the study just cited, from the majority of horses immunized for a period of more than a year serums with 500 to 1,000 units were obtained, and by the concentration of serum of lower potency, Cooper and her associates obtained preparations equal to or stronger than high-grade unconcentrated serums. In addition, the authors developed potent bivalent serums for those types which gave marked cross-agglutinative reactions.

The original publication should be consulted for full information concerning the incidence and severity of cases due to the different types, their presence in normal individuals and in spinal meningitis, and their virulence for mice. The original serological classification of Dochez and Gillespie as amplified and extended by the additions discovered by Olmstead and by Cooper and her colleagues covers practically all strains that have been studied and is now tentatively accepted as standard.

The occurrence of many of these pneumococcal types in Germany has been reported by Silberstein (1933),[1287] who found twenty-one of the new types, while there were only three strains which could not be classified. Types XVI, XXI, XXIII, XXVIII, XXX, and XXXII which had not before been reported in Germany were among those identified by Silberstein. Of all the types, XVIII and XIX seemed to be among the more common, as had earlier been noted by Gundel and Schwartz.*

That there may possibly be types beyond the present serological classification is suggested by the report of Christie (1934).[231] Of one hundred Group IV strains tested, forty-seven failed to react with specific serum for any of the thirty-two known types. Nineteen of thirty-six cultures from healthy persons, sixteen of thirty-six strains obtained from convalescent carriers, and twelve of twenty-eight organisms isolated from patients with acute pneumococcal pneumonia could not be identified by Christie with any of the type-specific serums. In India, Napier and Dharmonda[943] encountered among the pneumococci isolated from forty-five cases of lobar pneumonia and from fifteen cases of bronchopneumonia, strains that failed to correspond to any of the recognized types. Forty-six per cent of the strains studied apparently belonged to two types found only locally.

Recently two strains of pneumococci were described by Smith[1296] which the author claims constitute two new serological types. The organisms were isolated on separate occasions from the respiratory tract of a man suffering from "chronic bronchitis." A third but similar strain was found to be present in pure culture in the lungs of an individual dying of pneumonia. The strains grew anaerobically and were virulent for mice but, under the conditions tested, were not infectious to guinea pigs. The three strains appeared to represent two distinct serological varieties both of which differed from the thirty-two known types. There was no cross-

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* Quoted by Silberstein.

agglutination between the two varieties and, with the exception of one strain, that agglutinated with Type XVI immune serum in a dilution of 1 to 32, the two varieties failed to be agglutinated with any of the thirty-two type serums.

Strains of pneumococci which failed to correspond to any of the thirty-two known types were encountered in China by Wu and Zia,[1552] who reported that among a total of 162 strains tested, four failed to agglutinate with any of the thirty-two type serums. The unclassifiable strains possessed typical cultural characteristics and were virulent for mice.

It may be expected that other types of pneumococci will in time be added to Cooper's list. There were still a few strains which did not fit into any of the thirty-two groups, and the work of the two Japanese bacteriologists, while leaving doubts as to the soundness of their conclusions, and the results obtained in England, India, and China present the possibility that more heterogeneous strains may be encountered which will be found to form additional serological types.

There is the further question of the pneumococci causing infections in animals other than man. Grenier (1912)[555] described cultures from three sources, namely, guinea pigs, rabbits, and horses. The first two cultures from guinea pigs and rabbits were isolated from animals undergoing experiments with other toxic or infectious agents. The pneumococcus infecting the guinea pig was moderately virulent for mice and usually led a saprophytic existence in the respiratory and digestive tracts. The culture from infected horses did not originally kill mice but acquired virulence on animal passage. The observations of Grenier suggest that in veterinary bacteriology there may be strains, or even types, which might be found to have interesting pathogenic and serological relationships with pneumococci of human origin. The authors of the present volume disclaim any acquaintance with the literature of that branch of bacteriology, so far as it concerns Pneumococcus, but studies on

this phase of the biology of Pneumococcus, if they have not already been made, should be undertaken because of the additional information which may accrue.

Classification According to Electrophoretic Potential

Another criterion for classification, other than agglutination or protection tests, was advanced by Thompson (1931).[1396] By determining the electrophoretic rate of migration, he grouped the sixty-seven strains studied into five groups. The A group included typical Type III's and one Group IV strain. Group B consisted of typical Type I strains and a few Group IV strains. C took in Group IV strains, an atypical III, and two atypical II organisms. D was represented by a typical Type II, a few of Group IV, and one atypical Type I strain, while E was represented by only two Group IV strains. Since Groups C and D were shown not to be definitely distinct from each other and since the number of strains comprising Group E was too small to be significant, there remain only three large electrophoretic groups. In a second paper, Thompson[1397] presented data on the rate of migration in the electrophoretic field and the isoelectric points of various pneumococcal strains, but made his classification somewhat unwieldy by the addition of intermediary groups. While knowledge of the differences in the electrophoretic potential of different strains of pneumococci may be of scientific interest, the method has no advantage over the serological classification.

We are now learning of yet wider deviations from special specifications than those we have already discussed. At first sight they seem to confuse the definite lines of demarcation that have been drawn between bacterial species and the still finer distinctions that have been established on both chemical and serological grounds for types within the species. Pneumococcal cells possess polysaccharides peculiar to each type, and the carbohydrate is looked upon as the factor that determines the exact place of a Pneumococcus

within the species. Lately it has been found that other and apparently unrelated bacteria also elaborate complex carbohydrates, and that these carbohydrates both as antigens and as haptens exhibit immunological similarities to the polysaccharides of Pneumococcus. Puzzling as these new developments are at present, they, like the discovery of the transformative processes, will only lead to the disclosure of new biological principles.

There is no doubt that the members of other microbial species and materials of vegetable origin contain polysaccharides which may be found to possess chemical and immunological relations analogous to the capsular carbohydrates of Pneumococcus but, as Heidelberger and Avery concluded, this type of immunological correspondence in no way invalidates the systematic classification of bacteria based on the more usual and general methods of species determination, and it is of greater immediate significance in connection with the study of problems dealing with bacteria as disease-producing agents than in the study of bacteria in their generic relationships.

Type Determination

The immunity reactions which form the basis of serological methods for the bacteriological differentiation of pneumococci are of inestimable importance to students of Pneumococcus and to clinicians. They aid in defining the relations and interrelations of members of this bacterial species; they afford clues in tracing the spread of pneumococcal infection; they reveal the influence of chemical constitution in determining antigenic specificity and immune response; and have an eminently practical bearing on serum treatment.

Whatever benefit is to be derived from the use of antipneumococcic serum depends on the rapid and accurate determination of the type of Pneumococcus causing the infection. While the potency of serum and the judgment directing its administration are of great

importance, it is the use of the immunologically appropriate se-
rum and the promptness of treatment which in many cases may de-
termine the issue for the patient. The major effort, therefore, be-
sides increasing the accuracy of these tests, has been expended in
devising ways of shortening the time elapsing between the collec-
tion of the specimen and the identification of the serological type
of the infecting Pneumococcus.

MOUSE PROTECTION TEST

It was Neufeld and Haendel[991] who, in 1910, realizing the im-
portance of the delay involved in the isolation, cultivation, and
identification of pneumococci from infected material, saw in Unger-
mann's[1433] suggestion of testing cultures on the basis of phagocytic
action, possibilities for reducing the time factor. Ungermann's
plan was to inject a mouse intraperitoneally with sputum, then
several hours later to inject immune serum, and after a further in-
terval of an hour and a half, to kill the mouse and make stained
smears of the exudate from the surface of the liver. When the
serum corresponded immunologically with the culture, marked
phagocytosis of the cocci took place.

The use of the mouse as a vital differential medium cut short the
time usually required by plating and subculturing and became the
foundation of the protection test developed by Cole and his asso-
ciates.[86] This method, which is given in detail in the Appendix, im-
mediately came into general use, and while in the routine examina-
tion of pneumonic material it has given place to the more rapid
presumptive tests, it still remains the method of choice for the ulti-
mate determination of pneumococcal types. There are certain diffi-
culties encountered in the practice of this method, such as the
occurrence of pneumococci of more than one type in the specimen,
the overgrowth of Pneumococcus by other organisms in the peri-
toneal cavity of the mouse, the occurrence of cross-agglutination
when undiluted or slightly diluted immune horse serum is used, and

the complication arising from a latent mouse-typhoid infection in the test animals.*

Sutliff's[1359] experience emphasized the apparent errors which may occur when the testing of sputum by the mouse method is taken as the only diagnostic criterion. He compared the results of the protection test with those obtained by other cultural methods or, in other words, checked the type of Pneumococcus found in the sputum with the type or types isolated from the same patient by means of blood cultures, from post-mortem lung cultures, and from miscellaneous exudates. Among eighty-one cases in which Group IV strains were found in the sputum, twelve showed the presence of pneumococci of one of the fixed types in cultures obtained from the blood or lung. This error of 14.8 per cent is significant. Sutliff demonstrated the advantage in performing a type determination on cultures isolated from the hearts' blood of the test mice as well as on those from the peritoneal exudates. In 1,326 such examinations, the outcome was positive in fifty-five instances with the hearts' blood where the peritoneal exudate was negative. Moreover, Sutliff at times recovered two types of pneumococci from the same mouse. Of 339 cases where the peritoneal exudate showed a fixed type, the hearts' blood of the same test animals in fifteen, or 4.4 per cent of these instances, yielded an organism of Group IV and, in five, or 1.5 per cent of cases, pneumococci of a different specific type. In 862 cases in which the peritoneum of the mouse yielded a Type IV organism, the hearts' blood showed fixed types in thirty-one cases, or 3.6 per cent. Sutliff then, in all cases showing Group IV pneumococci in the sputum, collected and examined a second specimen by the mouse method and here the same discrepancy held. Of 145 cases, twenty-two gave results on the second determination inconsistent with those of the first. Sutliff's experience prompted him to advise that "When a specific type of

* Faber,[378] feeling that a substitute for mice was needed, recommended rabbits, but it is feared that he failed to appreciate the economic aspects of such a substitution.

pneumococcus is obtained from the sputum by mouse test, it may safely be considered the cause of the disease, but when a pneumococcus which does not react specifically (type 4) is found in the sputum, a second examination will in approximately 15 per cent of the cases yield a specific reaction for one of the fixed types."

Gundel[568] pointed out the various sources of error in the bacteriological diagnosis of pneumococcal infections. He recommended the examination of successive specimens of sputum, mentioned the difficulty presented by the presence of more than one type of Pneumococcus in sputum, and explained the replacement of organisms of the fixed types by those of the heterogeneous "X" (American Group IV) by assuming that virulent Type I and II organisms disappeared from the sputum as the pneumonic disease progressed, thus allowing a predominance of pneumococci from the upper respiratory tract.

The reports cited above are introduced to emphasize the need for thoroughness and care in the examination of infected material submitted for pneumococcal diagnosis. In some specimens, a single type of organism may be present and may so predominate that its identification by any one of the methods is a simple matter. When, however, pneumococci are few in number, or when variable results occur, the sputum should be examined by other methods and controlled by serological tests on cultures derived from single colonies.

In 1917, Blake[123] devised a method for obviating the difficulties in the mouse test. He injected the mouse with sputum and, when infection had sufficiently progressed, washed out the peritoneal cavity with sterile saline solution, centrifuged the washings at high speed, and to one part of the sterile supernatant fluid added an equal part of diluted immune serum. When the organism was of the same serological type as that of the serum a precipitate formed immediately. Used with a large number of strains, Blake found this test to be consistently positive and specific for Types I, II, and III. The principle of the method lay in the liberation of soluble

specific substance, or precipitinogen, from the pneumococcal cells during growth in the peritoneum of the test animal.

CULTURE AGGLUTINATION

In 1918, two rapid methods were announced. The urgency of war-time needs, the lack of laboratory facilities in the hastily improvised army camps, and the shortage of mice, all called for a simplification and speeding up of the methods for pneumococcal type determination. Taking advantage of the more rapid growth of pneumococci in sugar broth, Avery[84] planted washed sputum in bouillon containing one per cent glucose and after five hours' incubation, that is, in the period of active growth of pneumococci and before accompanying bacteria had multiplied to any conflicting extent, the broth was used as an agglutinating antigen with the three type serums. This procedure gave a high percentage of successful results, besides being simple, inexpensive, and speedy of execution. Oliver[1026] later made the suggestion that the Andrade indicator be added and that inulin be substituted for dextrose in the Avery medium, because these modifications yielded better results and were successful in cases when dextrose broth failed.

An exception to the general experience with the Avery method was that reported by Beckler, Marden, and Gillette,[97] who had a number of failures with this test as compared with mouse inoculation. Since they had no control over the collection of the specimens, the work being done in a state diagnostic laboratory, the sputum was often mixed with saliva, which the authors thought might account for their unsatisfactory experience. Those readers desiring to learn of the operation of the Avery rapid cultural method in the field should consult Vaughan[1448] who gave (1918) a description of laboratory requirements and satisfactory results in army base hospitals.

URINE PRECIPITATION TEST

Another diagnostic, or more exactly prognostic, aid of the same

nature was the urine test of Dochez and Avery,[321] who had ob-
served that *in vivo* as well as *in vitro*, Pneumococcus produced a
soluble specific substance, and that with an appropriate immune
serum its presence could easily be detected. Type III organisms
formed the largest amount of the substance, Type II somewhat
less and Type I the least. The majority of patients who failed to
show the precipitable substance in the urine recovered, whereas the
mortality was high among cases in which its presence was demon-
strable. A positive test was also of diagnostic significance.

SPUTUM PRECIPITINOGEN

A rapid method depending upon the demonstration of pneumo-
coccal precipitinogen was that of Mitchell and Muns (1917).[904]
They ground sputum with fine sand to disrupt the cells and release
the soluble substance, extracted the mixture with salt solution,
centrifuged the extract until clear, and then added immune serum
to the supernatant fluid. The test required only an hour for its
completion and gave clear-cut results for Type I, II, and III
pneumococci. Those sputums which gave no reactions were classed
as Group IV. The success of the method depends on obtaining a
specimen rich in pneumococci.

SPUTUM DIGESTION

Krumwiede and Noble,[761] also responding to the demands of the
time, sought by digesting sputum with antiformin to bring the pre-
cipitinogen into solution, but difficulties were experienced in apply-
ing this technique to all specimens, and the final results were often
unsatisfactory. In a second paper, Krumwiede with Valentine,[762]
abandoned the use of antiformin and, instead, coagulated the spu-
tum with heat. They broke up the coagulum and extracted the
soluble antigen with saline solution in a boiling water-bath, cleared
the extract by centrifugation, and then layered or floated the anti-
gen over the type serums. The authors incubated the tubes at 50°

to 55°, and observed a more or less opaque contact ring when the antigen and antiserum were of the homologous type. Here again there were some failures due to the quality of the sputum, but with the majority of specimens the method gave a rapid and accurate type diagnosis.

The Krumwiede method was given a trial by Kohn[737] with a limited number of cultures. The results, in the main, agreed closely with those obtained by the mouse-protection test. Because of accuracy, simplicity, and the saving of time and mice, Kohn advised its more general adoption.

A somewhat unusual case was reported by Gilbert and Davenport[514] which called attention to the complication presented by the occurrence in sputum of pneumococci of more than one fixed type. The Krumwiede test was negative with the three type serums. The culture in the Avery medium showed a faint reaction with Type I serum but none with that of Types II and III. A mouse inoculated with the sputum was dead at the end of forty-eight hours and the agglutination, precipitation, and cultural tests all showed the sole presence of Type III Pneumococcus. Direct planting of the sputum and of the Avery culture on blood plates yielded a predominance of green-producing cocci, which culturally proved to be Type I organisms and *S. viridans*. Serologically the growth agglutinated with both Type I and III serums.

Another avenue of approach was that reported by Loewe, Hirschfeld, and Wallach.[821] Instead of searching for precipitinogen in the excretions of pneumonia patients, they sought precipitin in the blood. The blood was drawn into potassium oxalate, laked with ether, and added to saline suspensions of type strains of pneumococci grown on glucose-serum agar. The mixtures were incubated until a color change due to alteration of the hemoglobin appeared. Inasmuch as immune substances do not usually appear in the blood stream in the very early stages of pneumonia, it is difficult to see how this method could possess any advantage over those aimed to detect the presence of precipitinogen in the urine or blood.

BILE-SOLUTION OF SPUTUM

In 1920 and 1921, Oliver[1025-6] reported a method by which the precipitable substance of the pneumococci in pneumonic sputum was brought into solution by bile and the filtered material then set up against known antiserums. With incubation at 42° to 45°, when the serum and the specific substance derived from the organisms were of the same serological type, immediate clouding and flocculation appeared, the whole procedure requiring only about thirty minutes for completion. Checked against the mouse-protection method there was full agreement in the results obtained.

The Oliver technique had advocates in Sharp and Urbantke.[1261] These authors in a study of forty cases found that when pneumococci of fixed types were intermingled with streptococci and Group IV organisms, the presence of the former was often obscured and identification difficult. The Oliver method appeared to be more successful than the classic mouse method with such specimens. Direct type determination of the infecting strain in pneumococcal meningitis was rendered feasible by slightly modifying Oliver's scheme. The sediment from centrifuged spinal fluids was treated with sodium taurocholate, the mixture shaken, and after being allowed to stand, centrifuged at high speed. The supernatant fluid was then mixed with type serums. A positive outcome was indicated by the rapid formation of a precipitate. The procedure could also be carried out on a microscope slide with equally definite results.

Rosenthal and Sternberg[1175] proposed another rapid procedure for type determination, which consisted in digesting sputum in a borax-boracic acid solution and adding to the clear supernatant fluid of the centrifuged mixture, in separate sections on a slide, specific antiserums. The results by this method agreed in the majority of instances with those obtained by the mouse inoculation test.

Instead of utilizing the presence of precipitin as a therapeutic control, Noble[1017] described a simple, though not particularly original, method for determining the presence of agglutinins in the

blood. The patients' serum was added to heavy suspensions of pneumococci. These materials used in small quantities and in concentrated form gave a rapid end result, and because of its reliability Noble used the method to measure agglutinins in the blood of pneumonia patients during serum treatment.

SLIDE AGGLUTINATION

In 1929, Sabin[1201] introduced his simple rapid "Stained Slide" microscopic agglutination test. Briefly, the test consisted in injecting the sputum into the peritoneal cavity of a mouse, then a few hours later puncturing the abdominal wall, withdrawing a small amount of peritoneal exudate, mixing it with the diagnostic serums of the various types in separate drops on the same slide, and finally smearing, fixing, and staining the mixtures. The slide was examined under an oil immersion lens for evidences of agglutination. Sabin also applied this method to the determination of antibody in the blood of pneumonia patients for the control of serum administration, as proposed by Park and Cooper.[1057] A drop or more of the patient's blood was withdrawn into a capillary tube, and after coagulation and contraction of the clot, the tube was centrifuged and a minute amount of the extruded serum spread with a loopful of a saline suspension of a heat-killed culture of the type for which agglutinins were sought. The film was air-dried, stained for one-half minute with fuchsin, and examined microscopically. When checked against the Noble and macroscopic agglutinin methods, this test was found by Sabin to be two and one-half to three times more sensitive when standard diagnostic type serums were used for comparison. In a paper published in 1930, Sabin[1202] gave further details and refinements of the technique. In addition to using the method for the control of serum administration, he found it to be of service in detecting the presence or absence of active infection in man.

Armstrong (1931),[22] giving Sabin credit for originating the method, introduced a slight modification of Sabin's technique by

mixing with droplets of peritoneal exudates from mice previously injected with pneumonic sputum loopfuls of diluted Type I, II, and III agglutinating serums. Instead of spreading and fixing the film, the material was immediately examined under a cover-slip. The differences in the two methods are minor and the selection of one or the other is a matter of personal choice. The important observation of Armstrong's was that an increase in the size of the cocci appeared after the addition of homologous serum—the significant *Quellung* effect first observed by Neufeld and later made the basis by Neufeld and Etinger-Tulczynska[987] for their rapid type-determination method. A year later, Armstrong[23] published a supplemental report on the results obtained by his method. In every case the type determinations were confirmed by mouse inoculation and other methods not specified. He also reported its applicability to cerebrospinal fluids, pus from empyemata, aural discharges, and exudates from various sources.

Logan and Smeall[823] slightly changed the Armstrong modification of the Sabin method, but the change was insignificant and the satisfactory results the authors obtained with it might well be credited to the original procedure.

Calder[196] devised another slight modification of the Sabin technique. The cultures grown in Avery's medium were dried on a cover-glass, stained with a drop of gentian violet solution, and after the addition of diluted type serums, the preparation was examined as a hanging drop.

Valentine,[1444] in 1933, introduced yet another variation of the Armstrong-Logan and Smeall modification of the original Sabin method. Here again, sputum and serum samples were mixed, although in a somewhat different manner, smears made and stained first with carbol-fuchsin and then carbol-thionin. The bodies of the cocci, when the immune serum was homologous for the strain, stained black while everything else on the slide was red, including the capsule. It is difficult to discover any advantage in this technique over the simpler methods.
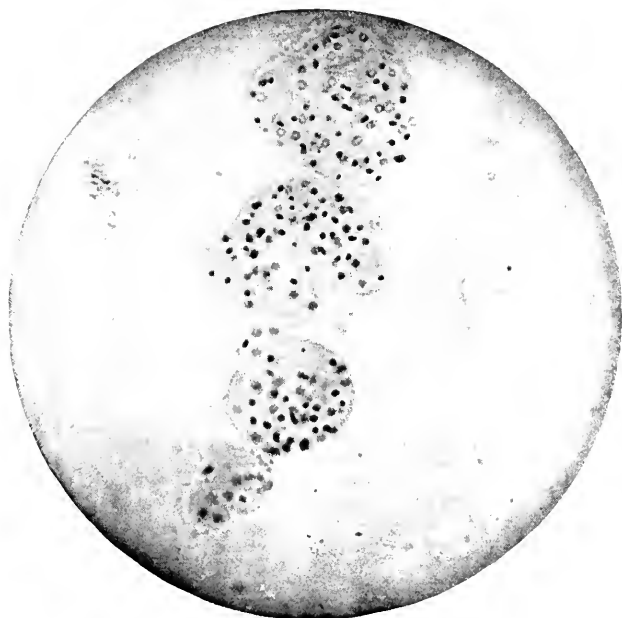
Figure I
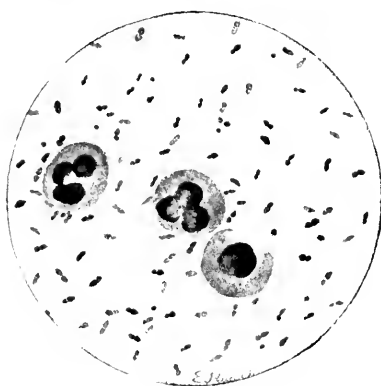
After Neufeld and Schnitzer[1000]

Figure 2

THE QUELLUNG PHENOMENON

Figure 1. Agglutination and *Quellung* of pneumococci by immune serum *in vitro*. Figure 2. Pneumococci from the peritoneal cavity of a mouse mixed with concentrated heterologous immune serum (control).

Figure 3
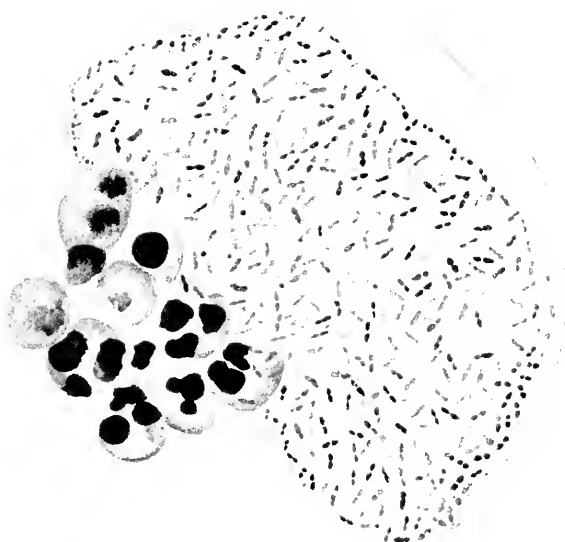
After Neufeld and Schnitzer[201]

Figure 4

THE QUELLUNG PHENOMENON

Figure 3. Pneumococci from the peritoneal cavity of a mouse mixed with concentrated homologous immune serum. Figure 4. Pneumococci from the peritoneal cavity of a mouse mixed with concentrated homologous immune serum; particularly marked *Quellung* and mass formation.

The Sabin method, either in its original or modified form, was for some time the one of preference. Among the many satisfied users of the original technique was Brown[151] of the Connaught Laboratories. Heffron and Varley,[602] reporting in 1932 the experience of the Bacteriological Laboratory of the Massachusetts Department of Public Health with the Krumwiede, Sabin, and macroscopic agglutination methods, preferred the Sabin procedure for routine examinations because of the greater number of positive results and the shorter time required. The types as determined by the Sabin method were in all cases checked by agglutination of the culture from the hearts' blood of infected mice. The simple technique was so readily learned by laboratory technicians that Heffron recommended its adoption by diagnostic laboratories.

Bullowa and Schuman,[185] for the purpose of still further reducing the time factor when a determination of any of the thirty-two types was desired, proposed the pooling of type serums other than I, II, and III. For example, combined lot A comprised Types IV through VIII, while B, C, D, and E included Types IX through XXIII.* With these lots, diluted with saline solution in a ratio of 1 to 10, 1 to 15, and 1 to 25, Bullowa and Schuman made preliminary tests and, obtaining a positive result in any group, then tested the strain separately against the monovalent type serums included in that group. These groups may be arranged to fit the prevalence of the various types in different communities or seasons.

At about the same time, Amoss[13] described another method. A sample of the patient's blood was defibrinated, the serum filtered through celloidin in a Coor's filter, and the filtrate centrifuged at high speed. The filtrate was then concentrated on a steam-bath under diminished pressure. To aliquot portions of the concentrate containing the soluble specific substance of Pneumococcus were added equal amounts of specific serum of the three fixed types, and during incubation at 37° in a water-bath readings were made in a

* Two other lots, F and G, could, of course, be added to include the remaining types.

beam of light at five-minute intervals. In some cases definite pre-
cipitation was noted in the homologous mixtures after five minutes'
incubation. To the remainder of the filtrate eight volumes of 95
per cent alcohol were then added, and, after mixing, the tubes were
set in the ice-box over night. The flocculent precipitate was col-
lected by centrifugation, the residue dried by heat under a stream
of air, and after salt solution was added, the solution was dis-
tributed into tubes for the addition of serum. The author tried still
another method for demonstrating the presence of precipitinogen
in patient's blood. To the serum collected as in the first procedure
distilled water and 0.2M sodium acetate-acetic acid buffer solution
with a pH of 4.6 was added. The mixture was then boiled until
coagulation was complete. The filtrate from the coagulum was
evaporated to dryness over a free flame, and heated carefully until
the odor of acetic acid was no longer present. The resulting dry
concentrate was extracted with sterile distilled water, the solution
cleared by centrifugation or by filtering through paper and the re-
sulting liquid used as a precipitating antigen against type serums
I, II, and III. The delicacy of this method was shown by the fact
that precipitinogen could be detected in the blood when blood cul-
ture remained negative.

To repeat Amoss' conclusion: "The method requires less time
than the sputum culture or mouse method, but has no advantage
over the sputum extract method. It is useful for typing in cases in
which neither sputum nor urine can be obtained"; to which might
be added, it furnishes to those who are not content with negative
or doubtful results one more aid in arriving at a type diagnosis.
Amoss also saw in this method, by detection of the soluble specific
substance in the blood, a means of judging the amount of serum
necessary and for following more closely the results of serum ther-
apy in lobar pneumonia. The intricacy of the procedure would bar
its use in the busy routine of the average hospital laboratory, but
it would be of service, where time and the facilities permitted, in

estimating the degree of absorption, distribution, and neutralization of injected pneumococcal antibodies.

Another variation of the precipitin method was that described by Sia and Chung (1932).[1271] They seeded cultures into poured plates of dextrose-beef-infusion agar containing, in separate Petri dishes, type-specific serums. After incubation, when the type of organism and serum corresponded, a well-defined annular opacity surrounded each colony, an appearance which proved to be type-specific. The method is obviously somewhat limited in value. It requires discrimination in the quantity of inoculum as well as in the amount of serum to be added and since, with these disadvantages, it also requires twenty-four hours' incubation and large supplies of immune serum, there is no reason for selecting it instead of the less complicated and more rapid methods. In the same year, Petri[1084] described a procedure which he used in the study of pneumococcal dissociation, but which, because of the recent development of simpler techniques, has not been adopted.

QUELLUNG PHENOMENON

The latest method to be generally used is based on the *Quellung* effect first described by Neufeld[974] in 1902. It promises to supersede all the previous rapid precipitation and agglutination methods. The technique as reported by Neufeld and Etinger-Tulczynska[988] consists in placing on a slide, separately, three loopfuls of the sputum to be examined, with two loopfuls of undiluted type-agglutinating rabbit serum on each drop of sputum, and then the same amount of Loeffler's methylene blue. Each drop of the mixture is covered with a cover-glass, and examined at intervals for five to thirty minutes through an oil immersion lens. When the type of the serum and of the pneumococcus are the same, the cocci, stained blue, are seen surrounded by swollen capsules. The capsules are a pale greenish-gray with a characteristic ground-glass appearance, and are distinctly outlined by a thin, dark line around

which appears a halo of light. When the serum and organisms are of different types, the pneumococci are of their usual size and are barely visible. This *Quellung* or swelling phenomenon is a distinct and specific reaction between the capsular substance of the pneumococcal cell and antibody of the same type. In the same year as that of Neufeld and Etinger-Tulczynska's publication, Sabin[1207] applied the method in the examination of sputum of one hundred cases of lobar pneumonia. The results with Type I and II organisms were found to correspond exactly to those of the mouse test. In two cases Sabin was able to identify Type I organism, whereas the results by older methods were negative. Furthermore the test was sometimes positive in purely salivary specimens.

In the next year (1934), after an experience of sixteen months, including two months in which they extended the use of the Neufeld test to all types including those from IV through XXXII, Beckler and MacLeod[96] confirmed the results by other methods in 96 per cent of the examinations. They unqualifiedly recommended this method because of its accuracy, simplicity, rapidity, and the small amount of sputum required.

Summary

The species *Diplococcus pneumoniae* has been divided by means of agglutination and other immunological methods into thirty-two separate and distinct serological types. There is evidence at hand that points to the existence of pneumococci that may belong to types other than those already established. Further investigation may reveal some new type identities, but the present list comprises the great majority of the members of this bacterial species. On the basis of cross reactions, there appears to be some relation between organisms of Types II and V, III and VIII, VII and XVIII, and XV and XXX, but the resemblances are not sufficiently close to invalidate the current classification. In nature there appears to be a stability of the types, although, as will be shown in the succeed-

ing chapter, transformation of a strain of one type into an organism of another type can be accomplished by appropriate treatment of the culture.

The particular serological type to which a pneumococcus belongs may be determined in several ways. The agglutination, precipitation, complement-fixation and mouse-protection tests, or combinations and modifications of these tests, may be applied to disclose the type identity of a strain, but it is safe to conclude that for routine needs the Neufeld method for determining specific types of Pneumococcus should be the one of choice. It gives an answer in the shortest time, possesses a high degree of accuracy, and is simple to perform. It makes for a saving in expense and eliminates the necessity of using mice. Whenever the results are not clear or when any doubt exists as to the type to which the organisms belong, the mouse-heart-blood or mouse-protection test should always be carried out for confirmation. Before hope of type identification is abandoned, the more refined methods such as that of Amoss should be tried. The usual cultural procedures, carbohydrate fermentations—especially of inulin—and bile-solubility tests are always to accompany the bacteriological diagnosis when uncertainty arises. For clinical diagnosis and for the direction of serum administration, as well as for epidemiological studies, laboratories should be prepared to run their cultures through the series of the thirty-two known serological types. The procedure requires a supply of potent, monovalent type-specific immune serums, preferably prepared by the immunization of rabbits with standard type strains. With type-specific serums, accurately prepared reagents, healthy laboratory animals, and careful bacteriological manipulations, there should be few cultures that cannot be serologically classified within the known fixed types.

PNEUMOCOCCAL DISSOCIATION AND TRANSFORMATION

Changes in the morphological, cultural, pathogenic, and immuno-logical characters of pneumococci caused by various physical, chemical, and serological conditions in their environment; the transformation of the diplococci from one serological type to an-other; and the relation of the species to streptococci.

THE constancy of the biological characters of Pneumococcus depends on the conditions of its surroundings. When the con-ditions are favorable, the morphological and serological integrity of the cell remains stable. Subjected to unfavorable influences, Pneumococcus exhibits great lability of form and function. The form of the cell may pass through every stage from the typical en-capsulated diplococcus to one completely denuded of capsule; pathogenicity may be diminished from full virulence to entire ab-sence of infectivity; and antigenic and serological properties may lose strict type-specificity and retain only the broader species-specificity. With the restoration of favorable conditions, the deg-radation process, if it has not proceeded too far, ceases and is re-versed, the cell again assuming its typical characters. But, what is still more remarkable, a degraded coccus originally derived from a fixed type may, under appropriate stimulation, develop the vital and immunological properties of a different specific type. Thus, in addition to natural occurrence of variation or dissociation, the actual transformation of pneumococcal types has been experi-mentally induced and, possibly, species mutation has occurred. Lack of knowledge of the existence of bacterial variants and of the factors inducing dissociation has undoubtedly caused much confu-sion in bacteriological diagnosis and in the interpretation of the serum reactions of Pneumococcus.

Early Observations of Dissociation: 1891–1921

Bacterial variation is not a new phenomenon. It is only the study and the explanation of the underlying causes that are recent. In 1891, Kruse and Pansini[763] first called attention to changes in morphology, cultural characters, and virulence of pneumococci under artificial cultivation. Pure cultures freshly isolated from pneumonic material were typical in appearance during early generations but, on continued cultivation on unfavorable media, the cells exhibited deviations from the normal characters. The lance-shaped, diplococcal forms became streptococcal or even bacillary, the capsule rapidly vanished, and virulence waned. The degenerated cocci, when passed through susceptible animals, regained their capsules and virulence, and when returned to favorable media, again showed normal pneumococcal morphology. Kruse and Pansini, therefore, noted many of the features of dissociation and were aware of the first causes of the phenomena.

In the next year, Arkharow[17] reported changes taking place in cultures cultivated in the serum of vaccinated animals. Growth was slow in developing and at the end of three or four days the cocci began to diminish in size, to grow in long chains, and to lose virulence. Four years later, Eyre and Washbourn[373] described the variations observed on continued cultivation of pneumococcal strains in broth. Of one strain it was said that it differed in "morphology, biology and pathogenic properties from the parent stock. It, in fact, represented a distinct variety, possessing practically no virulence, and growing luxuriantly, even at 20°C., on all the usual media." The first attempts to induce this variant to revert to its former state were unsuccessful. Then, by passage through a rabbit, the variant reverted to its original form.

Hiss, Borden, and Knapp (1905)[651] encountered organisms, indistinguishable in fermentative reactions from pneumococci, which showed variations in morphology or agglutination, and the authors considered that the cultures were temporarily or perma-

nently modified pneumococci, *Streptococcus mucosus*, or strepto-cocci of hitherto unrecognized types.

A suggestive communication, because of its anticipation of later discoveries, was that of Rosenow[1163] published in 1910. He pre-sented the results of a study of seven cultures isolated from endo-carditis which he believed were "modified pneumococci." All the strains fermented inulin and produced a variable amount of green-ish discoloration but no hemolysis on blood-agar plates, but grew atypically with the development of involution forms on media con-taining the patient's blood. However, cultivation in normal serum or blood and animal passage promptly restored normal pneumo-coccal characters to the modified strains. Recultivation in the pa-tients' serum brought out the modified characters. These special characters varied greatly in the strains studied; the more chronic the disease process in the patient from whom the serum was ob-tained, the more marked were the changes. This last observation would seem to argue for the occurrence of variation or dissociation *in vivo*—a biological process concerning which there is still some doubt.

The phenomenon of bacterial dissociation, or as it was known in the early years of this century, variation, received little atten-tion until 1915 when Friel[494] reported that prolonged cultivation of bacteria rendered them more susceptible to phagocytosis. He called the process "Piantication" (fattening for slaughter) and observed its operation with strains of Friedländer's bacillus, *Pas-teurella*, and Pneumococcus. The same effect was produced by ex-posing the organisms to immune serum; while the reverse process took place when "pianticated" bacteria were grown in normal se-rum—they regained their resistance to phagocytosis.*

In the next year, Stryker[1348] described the variations induced in

* Neufeld and Schnitzer credited the first demonstration of bacterial dis-sociation to Friel, although Hadley[584] ascribed the discovery to Baerthlein (1912). The phenomenon had, however, been observed much earlier by Kruse and Pansini (1891),[763] and by Arkharow (1892),[17] and had been described in some detail by Eyre and Washbourn[373] in 1897.

Pneumococcus by cultivation in immune serum. When virulent strains were grown in broth containing homologous immune serum, there developed variations in agglutinability, decrease in virulence, inhibition of capsule formation, increase in phagocytability with normal serum, and a change in absorptive power and in antigenic properties. Reversion of the changed forms to the original type took place on animal passage, the number of such passages required usually varying with the number of previous serum treatments. The immune response, as measured by agglutinins, was slower in rabbits injected with strains grown in immune serum than with those cultivated in normal serum. A spread of agglutinative action was evident in the ability of the serum of immune rabbits injected with a serum-treated Type II culture to agglutinate pneumococci belonging to both Types I and II. Type-specificity was being lost and replaced by species-specificity. Cultures grown in normal serum formed capsules upon injection into mice, whereas those grown in homologous immune serum under similar conditions showed no demonstrable capsules. This loss of the ability of Pneumococcus to synthesize the capsular substance was later to assume a new and broader significance.

Later Observations of Dissociation

SMOOTH AND ROUGH FORMS OF PNEUMOCOCCUS

Arkwright (1921),[18] in studying the colony appearance of dysentery bacilli grown on media containing immune serum, gave the designations S and R—smooth and rough—to the dissociants because of the corresponding differences in colony topography of each form. Griffith,[560] in 1923, extended Stryker's observations and applied the letters S and R to the two forms of colonies he observed when pneumococci were grown in media containing homologous immune serum. The S colonies have a smooth surface, and the cocci forming them produce the soluble specific substance in broth culture, agglutinate with specific serum of the homologous

type, are virulent for laboratory animals, and on injection into rabbits stimulate the production of type-specific immune substances. The R colonies have a rough surface, and the organisms comprising them form no soluble specific substance in broth culture, agglutinate atypically, and are avirulent. Cocci of the R colonies may revert to the S form, or they may remain stable for generations. Another property of the S cells is the ability to absorb from immune serum all antibodies for both S and R forms. The R forms absorb only the anti-R bodies, and when injected into animals fail to stimulate the formation of type-specific (S) antibodies.

Griffith considered that the R form was differentiated from the S by the loss of virulence and by the ability to form capsules and to elaborate soluble specific substance, and that the R form represented a stage in the degeneration of Pneumococcus from the virulent, complex type of S cell to an attenuated form with a simpler antigenic structure. Griffith also found that degradation might not be permanent and that reversion could take place after animal passage or repeated cultivation in blood broth. The author recommended for the demonstration of variant colonies an opaque "chocolate" agar to which red blood cells treated with chloroform had been added.

Griffith looked upon the S form as the original, unchanged organism, the R form as a variant due to unusual growth conditions. The degenerative action of immune serum Griffith believed to be a double one. He suggested as an explanation of the change the view that serum might disorganize the biological functions of Pneumococcus by precipitating the capsule, thus inhibiting the secretion of antileucocytic substances and rendering the organism temporarily harmless, and that when pneumococci divided in the animal body in the presence of immune serum, the influence of the serum might cause progressive attenuation of subsequent generations. In connection with the causes for such bacterial variations, Eastwood[344] contributed an interesting theoretical discussion. It would

take us too far afield to quote from it here, but the communication is recommended to those readers who desire to learn more of the philosophical aspects of the phenomenon of dissociation.

In seeking a medium that would emphasize the differences in variants, Sia and Chung,[1270-1] by the substitution of normal dog blood for rabbit or horse blood in agar for plate cultures, obtained such marked differences in the morphology of S and R colonies that differentiation, they claimed, became extremely simple. With a moderate degree of magnification ($\times$ 28), the S colonies were seen to be smooth and glossy, while the R colonies, including those derived from Pneumococcus Type IIS, revealed a wrinkled and coarsely rough surface. The R colonies also exhibited strong hemolytic properties. Sia and Chung tested the blood of guinea pig, white rat, chicken, and cat, but none was so good as dog blood. These authors believed that the property of dogs' blood resided in the cellular elements rather than in the plasma, and probably was due to hemoglobin.

MODIFICATIONS A, B, AND C

Blake and Trask (1923),[129] in conducting experiments similar to those of Stryker, also found that growth of pneumococci in homologous serum resulted in a marked loss of virulence, accompanied by constant and distinct changes in agglutinative properties with respect both to the character of agglutination and the zone of optimal reaction. The authors found the changes not to be a gradual alteration of all members of a culture but, instead, there appeared to be a comparatively rapid and complete change in individual organisms. The variants exhibited changes not only in virulence and agglutinability, but also in colony appearance, by means of which three modifications, called by the authors A, B, and C, could be distinguished.

In the same year, analogous observations were published by Yoshioka.[1564] Typical pneumococci underwent apparently regular serological modifications when maintained under unfavorable con-

ditions, such as surface cultivation on unsuitable media, incubation at 39°, and too long-continued drying. The same conditions also led to loss of virulence. The changes noted consisted in a marked decrease in agglutinability with homologous serum and in the appearance of an enhanced agglutinability with heterologous serums. The modified strains were, at times, agglutinable by anti-streptococcic serum. The changes appeared irregularly and suddenly and did not parallel the degree of decrease in virulence. An immune serum obtained after immunization with an atypical strain agglutinated only that specific variant and not the parent strain.

In the discussion which followed the presentation of papers on bacterial variability before the German Association of Microbiology at Göttingen in 1924, Neufeld[979] reported a change in bile-solubility of pneumococcal variants, as well as in their susceptibility to optochin. In the same year, Felton and Dougherty[425] observed that pneumococci when grown in plain broth in an automatic transferring device suffered a loss of virulence which was directly proportional to the change in the hydrogen ion concentration of the medium—the more acid the medium the greater the loss of virulence. Accompanying the change in virulence there was an alteration in the behavior of the organisms toward agglutinating serums. Although specific, the agglutinability of the modified strains became greater than that of the parent organism.

COMPOSITE CULTURES

Amoss[12] in 1925 published an article on the composite nature of a pure culture of virulent pneumococci from which he derived several strains by the Barber single-cell technique. These were cultivated in broth containing Type I antiserum, and the pure culture was submitted to successive transfers in bile broth and acid broth. Amoss reported that the virulent strain of Type I Pneumococcus, after being passed through 190 mice, was composed of individuals possessing characters differing from those of the original culture. A pure-line strain derived from a single cell isolated from a viru-

lent composite culture was more virulent for rabbits and less re-
sistant to unfavorable media than was the composite strain or
other strains similarly obtained from the same source. Amoss iso-
lated by the plating method an avirulent strain from the compos-
ite virulent culture which had been repeatedly transferred and
grown in immune serum broth, bile broth, and slightly acid broth.
Cultures of the virulent single-cell derivative, when grown in these
media, also gave rise to the avirulent form. Heterologous immune
serum and also normal serum did not favor the change from viru-
lent to avirulent variants. The avirulent strains, however pro-
cured, were all of a single sort. They formed characteristic colo-
nies, showed no tendency to revert to the parent type, and did not
become virulent on repeated passage through mice. Serum from
rabbits immunized with the avirulent variants possessed aggluti-
nins but no protective antibodies for the parent strain. It seems
clear from Amoss' experiments that he had succeeded in effecting a
permanent degradation of a virulent Type I Pneumococcus, with a
loss of type-specificity, but not of species-specificity.

The results of Reimann's[1125] study, published in the same month
in which Amoss' publication appeared, agreed with both Amoss'
and Griffith's observations. Reimann reported that cultures of
pneumococci from single-cell seedings, when grown in broth con-
taining immune serum, bile, or even normal serum, suffered a de-
crease in virulence and loss of type-specificity. The changes might
take place when the pneumococci were repeatedly grown in plain
broth or on blood agar, but were due to variations in individual
cells, rather than in the cocci of the culture as a whole. Reimann
preferred 2 per cent unheated blood agar to Griffith's chocolate
agar, and on this medium there appeared characteristic smooth
colonies along with others of the rough form. Cultures from S colo-
nies were highly virulent, had large capsules, produced soluble
specific substance, dissolved in bile, and were strictly type-specific.
Strains from R colonies were avirulent for mice, had no capsule,
produced no soluble specific substance, did not dissolve so readily

in bile, and had largely lost their type-specificity. Single-cell cultures propagated from S colonies, after repeated transplants under unsuitable conditions, produced some R variants, while single-cell cultures from R colonies, under the same circumstances, remained constant in character.

SPECIES-SPECIFICITY OF ROUGH FORMS

A few months later, Reimann[1126] published these further conclusions:

Immune sera prepared with the degraded or variant forms of pneumococci (R strains) are similar in their reaction to sera prepared with the protein or cell solutions of pneumococci. They contain antibodies reactive with the protein of all types of pneumococci, but no antibodies reactive with the type-specific substances. Pneumococci of the variant or R form, regardless of type derivation, are serologically identical and have the antigenic characteristics of pneumococcus protein. They evoke the species-specific and not the type-specific antibodies. Antipneumococcic sera produced by immunization with S strains may contain species-specific antibodies in addition to those which are type-specific. Each kind of antibody can be removed separately from these sera by selective absorption with the R and S strains of pneumococci.

These fundamental observations were later to be confirmed and explained by the discoveries of Avery and Heidelberger of the antigenic chemical constituents of the pneumococcal cell.

Takami,[1373] contemporaneously with Amoss and Reimann, added a few new facts about variation. His study included certain strains that were apparently stable in their original characters, since they gave rise to no variants even after two or three years' cultivation. There were other strains that showed a strong tendency to vary, and in a short time became so changed that they no longer produced any typical colonies. In agglutinative abilities the same rule held true. There appeared to be no direct relation between decrease of agglutinability and atypical colony formation. The only two characters that were closely connected were bile-solubility and

inulin fermentation. When either of these properties was lost the other disappeared.

Takami[1375] followed the *in vitro* experiments with a study of the variations displayed by pneumococci propagated in the animal body. Rabbits, guinea pigs, mice, white rats, and house rats were used for this purpose, and the variants produced in these animals differed in agglutinative characters from the forms developed on artificial media. The explanation offered was that in the body the organisms lose their old receptors and acquire new ones. Takami separated five typical strains of "culture-bacteria" (pneumococci long grown on blood agar) into colonies that were still markedly agglutinable, and into others that had lost this power. The latter were found to be highly virulent for mice, whereas the former were avirulent.

A few years later, Kimura, Sukneff, and Meyer[711] repeated the dissociation experiments, using broth containing 10 per cent homologous immune serum, with subsequent cultivation of the variants on Griffith's chloroform-blood agar and Bieling's blood-water agar, both of which have a laked-blood base. The results were similar to those reported earlier, but the authors believed that they had demonstrated the production of other variants in addition to the atypical R forms with divergent cultural and serological characters. The experimental data, however, are insufficient for judging the claim.

For determining the true character of normal strains and of dissociants, Schiemann[1225] adopted as a criterion the possession of a type-specific (dominant) hapten as a prerequisite for the formation of type-specific agglutinins and protective antibodies and also for virulence. For the recognition of type-specificity the essential considerations were, first, coarse agglutination in homologous antiserum determined by the carbohydrate nature of the hapten; second, the repression of cross-agglutination in heterologous serum; and, third, mouse virulence. According to these standards, in

addition to normal and degraded R forms, Schiemann postulated intermediate variants which he claimed represented pseudo-types. The discussion was largely theoretical, and since he gave no experimental data, it is impossible to judge the validity of his claims.

ELECTROPHORETIC POTENTIAL OF VARIANTS

Falk, Jacobson, and Gussin,[383] and then Falk and Jacobson,[380] studied another criterion for variability. The authors measured the electrophoretic potential of Blake's variants A, B, and C from Type I Pneumococcus during cultivation on blood-agar slants, with weekly to bi-weekly transplants, over a period of one and one-half years. The velocities remained constant and paralleled the virulence of the strains. Although the authors believed that electrophoretic potential was related in some fundamental manner to virulence, phagocytability, agglutinability, and other serological characters of microorganisms, the particular variants studied were indistinguishable from the parent strain in these characters, and after a large number of generations on blood agar showed no evidence of spontaneous changes. The only exception to this stability of character was a single-cell strain of variant C which reverted to the A form on passage through a mouse.

EFFECT OF CHARCOAL, YEAST, OPTOCHIN

The variation in pneumococci appearing after growth in broth containing animal charcoal or dry yeast and subsequently in optochin broth, first observed by Berger and Englemann[100-1] and shortly afterward by Morgenroth, Schnitzer, and Berger,[929] was corroborated in 1927 by Amzel.[14] Cultivation in these media gave rise to rough colonies, the members of which were avirulent for mice and exhibited diminished solubility in bile. One strain developed hemolytic properties, and another became agglutinable with antiserum for the fixed types. The variations observed after cultivating the cocci in the presence of bile were the same as those occurring in the Schnitzer-Berger medium.

In Amzel's[15] next paper it was reported that pneumococci iso-
lated from pneumonia patients before optochin injections were of
the smooth type, while the organisms cultivated after injection
grew as rough colonies. Untreated cases yielded only smooth colo-
nies and, in two cases repeatedly treated with optochin, the iso-
lated culture was persistently composed of both smooth and rough
forms. Amzel attempted to convert the rough into smooth strains
by mouse passage but was able to effect this reversion in only one
of three trials.

During the 1920's there came abundant confirmation and expan-
sion of the earlier observations on pneumococcal dissociation. Ja-
cobson and Falk (1926–1927),[674-5] continuing their earlier studies,
were able to degrade smooth strains of Blake and Trask's A, B,
and C modifications into rough strains by growing the organisms
in broth containing specific immune serum, although after twenty-
three transfers the conversion was incomplete. The cultures were
still mixtures of S and R varieties. The former continued to have
the same virulence and electrophoretic potential, but the latter
were reduced in both virulence and potential. Rough variants of
the B and C strains reverted after twelve transfers in homologous
immune serum broth, and showed the same virulence and potential
as the original smooth organisms. In all the strains studied there
were alterations in virulence accompanied by parallel alterations
in electrophoretic potential and by reciprocal changes in agglu-
tinability. Levinthal[800] also observed changes in virulence and in
the cultural and serological behavior of a highly virulent Type I
pneumococcus after cultivation in serum broth. He was able to
effect the transformation of R to S forms by growth in broth at
25° and by subsequent mouse passage.

IN VIVO VARIATION

Similar variations apparently taking place *in vivo* were de-
scribed by Wadsworth and Sickles.[1474] Cultures isolated directly
from the blood stream of horses undergoing immunization, or at

the necropsy of animals that had died as a result of the develop-
ment of pneumococcal lesions in the heart or other organs, exhib-
ited attenuation of virulence, loss of capsule formation, antigenic
power and type-specificity, and changed susceptibility to phagocy-
tosis. In the case of some of these variants the specific characters
of the original type from which they were derived were quickly re-
stored by one or two mouse passages. In other instances, the or-
ganism remained atypical.

Sickles,[1279] in a later study (1932) of pneumococcal strains that
had become atypical in the tissues of horses undergoing immuniza-
tion, in comparison with the typical cultures from which they were
derived and with various other typical and atypical strains, found
that all the organisms were bile-soluble. The maximal limits of
growth, along with the other characters, such as limiting hydrogen
ion concentration and peroxide and hemoglobin formation, were
similar for the same type culture whether original, degenerated, or
reverted. Sickles found only one strain which departed from the
general rule and that organism grew at 42°, and survived even
after incubation at 43.5°. No other pneumococcal strains studied
were alive after twenty to twenty-four hours at 42°.

That Pneumococcus may, however, retain its specific type char-
acters when growing in the animal body was proved by Megrail
and Ecker[888] in 1924, who injected mice and rats with suspensions
of gum tragacanth followed by a saline suspension of pneumo-
cocci. In these fixation abscesses the strains displayed no variation
and no change in agglutinability. Here the conditions differed from
those in the horses harboring pneumococci, as reported by Sic-
kles,[1279] since the rats and mice had not been subjected to any im-
munizing treatment, and their tissues, therefore, presumably con-
tained no antibodies which might favor variation.

Reimann[1128] found that R forms occurred *in vivo* but could dis-
cover in his experiments no positive evidence that recovery from
pneumococcal infection depended upon the degradation of virulent

S forms to avirulent R forms with their subsequent destruction by phagocytosis. In a still later study[1129] it was noticed that daughter colonies frequently appeared among the R variants, and in some instances tended to replace the typical R forms. The daughter strains grew in colonies with glistening surface, morphologically indistinguishable from genuine S colonies, although the characters of the bacteria comprising the daughter colonies conformed to the R variety. Strains of R pneumococci, which had seemed irreversible, were apparently converted into the S form when treated by the method of Griffith, that is, by growth in specific immune serum. Reimann considered that recent experimental studies indicated that virulent S pneumococci might dissociate into the R form *in vivo*, that R forms occasionally could be found in the sputum of pneumonia patients, and also might live dormant *in vivo* for a considerable period of time.

The recovery of R variants from the body has recently been reported by Shibley and Rogers.[1262] Twenty-four lung punctures made in lobar pneumonia patients at the time of crisis or lysis yielded R forms of pneumococci in all but four cases.

DETAILS OF COLONY FORMATION

Dawson[299] maintained that colony morphology alone could not be considered as a final criterion of dissociation; it should be confirmed by specific agglutination and virulence tests. While it was possible, by mouse passage, to accomplish a complete reversion of Type IIR to Type IIS, it was not possible to convert the particular Type IR strain studied to the corresponding S form. In the case of a Type IIIR strain, it required twenty-eight mouse passages to restore the variant to its original S condition. Growth of the same R strains in broth containing 10 per cent anti-R serum resulted in reversion of Type IIR on the fifth transfer, of Type IIIR after eight to twelve transfers, but failed to affect the Type IR. Dawson thought that the reversion of R to S did not depend

on the presence of an admixture of both forms within the culture, but rather that each individual R strain might or might not possess the ability to revert. This varying tendency of R strains was exemplified in one experiment in which one of four other strains of Type IR, obtained by growing a freshly isolated Type IS strain in 25 per cent Type I anti-R serum, reverted to Type IS after forty transfers.

The finer details of colony appearance of R and S forms interested Paul (1927),[1067] who chose a small number of standard R and S strains and studied their growth on agar under a limited number of cultural conditions. Paul described the R colonies as having a rough surface, with a gradual and progressive increase in size over a period of several days and a tendency to remain discrete. The colonies failed to undergo rapid autolysis in early generations and exhibited limited secondary colony formation. Methemoglobin formation was present but might be replaced by slight hemolysis. Paul's S strains grew in rapidly developing disc-shaped colonies with a smooth surface which later showed irregularities. The colonies tended to become confluent and exhibited marked autolysis in thirty-six to ninety-five-hour cultures. In the same period, secondary colony formation took place. Methemoglobin formation was a constant feature.

In a second paper, Paul[1068] gave further information concerning the conditions which affected colony formation. Under extreme crowding, the individual S colonies gave way to irregular, amorphous, slightly elevated masses with myriads of tiny structures having irregular and roughened surfaces, comparable to R colonies, but on transfer to less crowded conditions they developed as typical S colonies. The true R colonies tended to remain discrete, but in dense growth resembled the S colonies under similar conditions. The effect of age on the S colonies was to increase autolysis and papilla formation. With the R colonies there was no autolysis, but roughness, opacity, and compactness became emphasized, with

papillae appearing on about the fourth day. When the blood con-
tent of nutrient agar fell below 5 per cent, the S colonies ap-
peared small and rough, yet were not true R colonies. The same
effect was brought about by an alkaline reaction of the medium,
but the original characters were restored on transplantation to a
more favorable medium.

In a study of the bile-solubility of Pneumococcus, Atkin[29] re-
ported that pneumococci growing in papillae or secondary colonies
developing on an autolyzed colony from a point inoculation on a
thick serum-agar medium of proper reaction were insoluble and
that susceptibility of the variants to the action of bile corre-
sponded to the possession by the organisms of autolysin. When the
insoluble cocci were subcultured on a fresh serum-agar slant, they
regained autolytic properties and bile-solubility.

Grumbach[563] also studied the details of colony formation accom-
panying the varying degrees of pneumococcal dissociation. He dif-
fered with Atkin,[29] but agreed with Paul that daughter colonies
were not identical with R forms of pneumococci, because they were
never truly granular on ascitic agar, they remained bile-sensitive,
were fully virulent, and on transplantation developed into "bud-
carrying" S colonies. Grumbach found that the ability to produce
hemolysis on blood agar and in blood bouillon in forty-eight to
seventy-two hours quite commonly ran parallel with the dissocia-
tion phenomena. He described the characters of three virulent S
strains isolated from pneumonic material that were not aggluti-
nated by Type I, II, or III serums. Growing for twenty-four
hours on ascitic agar the organisms produced the classical picture
of pneumococcal colonies. The thickness of the peripheral ring va-
ried, and in one case there was a "wall" formation of the type
Buerger and Ryttenberg[169] claimed to have found solely in colonies
of streptococci. Grumbach also described a "wing-form" colony
which he believed to be similar to that supposed to be caused by
phage action on streptococci, and concluded that the same colony

pictures could be obtained for pneumococci as for streptococci, but was not sure how far the bactericidal action of the body fluids or how far bacteriophagic action were to be considered as the basis for the phenomena.

Faragó (1932)[390] investigated the possible participation of bacteriophagic action in the dissociative processes, but decided that it was not a factor. He objected to the designation R and S for dissociants, because secondary colonies were formed from virulent organisms, whereas Griffith's R modification arose from avirulent strains. It is difficult to follow Farago's reasoning, but it may be possible that he had in mind some of the features later described by Dawson.

ANTIGENICITY OF ROUGH FORMS

Tillett[1406] turned his attention to the antigenic properties of the dissociated R forms. When he vaccinated rabbits by repeated intravenous injections of suspensions of heat-killed R pneumococci, the animals acquired a marked degree of active immunity to infection with virulent S forms of Pneumococcus I and II. (Tillett[1404-6] had previously shown that a similar immunization treatment induced active resistance to Type III infection.) Furthermore, the whole citrated blood of the immune rabbits passively protected normal rabbits against infection with Type I and Type III pneumococci, but failed to confer a like protection on mice. According to Tillett this form of acquired resistance to pneumococcal infection elicited by R organisms devoid of type-specificity, and exemplified in animals whose serum possessed no demonstrable type-specific antibodies, presented features which strongly suggested that the underlying mechanism differed from that concerned in type-specific immunity.*

RESPIRATORY CAPACITY OF VARIANTS

Another difference in the character of S and R forms was the

* For a full discussion and bibliography of microbic dissociation up to that time the reader is referred to Hadley's[584] comprehensive article.

changed respiratory capacities of pneumococcal variants. Accord-
ing to Finkle's[440] measurements, the capacities of organisms of
Types I and II were altered during conversion from the S to the R
form. For Type I Pneumococcus it was increased 110 per cent,
while for Type III it was diminished by 45 per cent. In the case of
Type II there occurred a diminution of only 16 per cent in re-
spiratory activity. At the same time, anaerobic glycolysis was in-
creased on the average 25 per cent each for all R forms irrespec-
tive of type derivation, while Type I Pneumococcus, on being
converted to the R form, lost its capacity for aerobic glycolysis.
Pneumococcus III in passing to the degraded stage gained this
activity, which is in accordance with the respective increase and
decrease in respiratory activity of the two types. In order to ap-
preciate the degree of the respiratory capacity of pneumococci,
Finkle stated that the O_2 consumption was for Type I pneumo-
cocci thirteen times and for Type II strains thirty-four times that
of the human tubercle bacillus (strain H_{37}). When compared with
the oxygen consumption of animal tissues, Type II strains con-
sumed over twenty times as much oxygen as did isolated rat kid-
ney tissue, and almost one hundred times as much O_2 as isolated
dog muscle.

A respiratory phenomenon connected with loss of virulence has
been described by Sevag and Maiweg.[1258] A virulent pneumococcus
on being transformed into its avirulent form consumes many times
more oxygen than the parent organism, but the gain of activity is
a temporary property. After a time, the avirulent variant degener-
ates into a form that consumes much less oxygen than either the
virulent or the recently derived avirulent form. The phenomenon
may be associated with the change in the structure of the enzyme
responsible for carbohydrate biosynthesis during the shift from
the virulent to the avirulent state and hence may be related to cap-
sule formation. According to Sevag and Maiweg, the addition of
colorless, clear, blood catalase or of a small amount of sodium
pyruvate to the culture enables the organisms to carry on their

respiratory functions and to maintain their reproductive capacities and virulence for a longer period of time.

Petrie (1932)[1084] suggested one more means for the identification of R and S variants. In stab cultures in agar plates containing 5 per cent immune serum the virulent S pneumococci grew with a distinct halo about the colony when the organism and immune serum corresponded in type-specificity. The halo apparently consisted of a specific precipitate formed by the interaction of the pneumococcal polysaccharide and the precipitin in the homologous serum. The R colonies, in contrast, produced only a faint and narrow halo after a considerable period of incubation. Serum from immune horses appeared to be more suitable than serum from immune rabbits for halo production.

INTERMEDIATE FORMS

In addition to the well-known S and R forms, Klumpen (1932)[780] mentioned intermediate forms growing in colonies designated as SU and RK. In other characters, however, the strains were either true S or R forms. Klumpen recognized the *Flätterformen* described by Grumbach, and noted that the organisms comprising daughter colonies were of the smooth type.

Still other variants intermediate between the S and R forms were derived from pneumococci by Blake and Trask (1933).[130] By growing Type IS pneumococci in homologous immune serum broth, the progressive appearance and disappearance of forms differing from both S and R cocci were observed. The forms were designated as a, b, c, d, and e. Two of the intermediates, Type Ib and Type Ic, were easily stabilized in pure culture. All showed an orderly change in agglutinative reactions in homologous and heterologous immune serum, and also underwent a progressive loss of virulence for mice. Blake and Trask produced only one intermediate form from Type IIS and none from Type IIIS.

The importance of recognizing intermediate variants in the dissociative process was emphasized by Paul[1069] of Blake's labora-

tory. He believed that two methods of inducing degradative dissociation in S forms seemed to give rise to the different patterns of variant production. Thus, when S forms were grown in homologous antiserum they became rapidly stabilized as R forms, but when S forms were cultivated in media containing bile, the S organisms displayed a greater tendency to become stabilized as c forms. Paul showed that during the reversion of c, d, and R forms, induced by growth in anti-R or plain rabbit-serum broth, intermediate variants arose in the reverse order to that in which they appeared during the degradation of S forms. The intermediate variants tended to become stabilized as b forms, which was the usual high level to which these strains reverted by this method.

A process possibly related to that studied by Blake and Trask was reported by Eaton,[345] an associate of Blake, who described the production of stable strains of Pneumococcus which underwent rapid lysis or failed to grow at 37°. For the strains he introduced the term "phantom colony" or "P–C" variants. This P–C variation, he claimed, was a change independent of the ordinary smooth-to-rough variation. Eaton, moreover, made direct isolation of these variants from cases of human infection.

Another apparent complication in the symbols employed to identify pneumococcal variants is to be found in the recent papers by Eaton (1934–1935).[345-6] In addition to the phantom colony or P–C variants, he observed smooth variants arising in the daughter-colony dissociation of stock smooth strains after incubation on blood agar at 25°. These smooth variants, called V, and the smooth parent strain, termed N, from which the former were derived, had the same virulence for mice and did not differ in antigenic composition as determined by agglutination, agglutinin-absorption, and mouse protection tests. The smooth V strains were stable, and while they, too, formed daughter colonies they dissociated to rough forms much less readily than did the N or freshly isolated strains. The N and V strains appeared to differ in their capsular staining reactions, and in the ability to form methemo-

globin in blood. Without an actual visual comparison of these V variants with the principals and intermediates described by other authors it is impossible to assign them their proper place in the dissociation order.

Further study is required before giving an estimate of the significance of these possibly new forms, although there is already much evidence to support the concept of a polyphasic cycle in bacterial dissociation.*

REVERSAL OF DISSOCIATION

Griffith[562] was successful in reversion experiments in the animal body. Some R strains which had not entirely lost their soluble specific substance readily reverted to the S form when passed through the mouse. The author obtained smooth colonies, with restoration of virulence and original serological type characters, after making massive injections of R strains into the subcutaneous tissues of the mouse. The original change from S to R forms was accomplished by ageing the colonies on chocolate blood agar containing horse serum and by cultivation in broth to which specific immune serum had been added. The greater the concentration of immune serum, the more complete and permanent was the change to the R form.

The possibility of the reversal of the dissociation process attracted Dawson and Avery[304] who, by mouse passage, not only brought back to the S form seven or eight cultures of single-cell isolation, pure-line S strains of Types I, II, and III, but also succeeded in causing six pure-line R strains to revert to type-specific forms by growing the cultures in media containing anti-R serum.† The authors failed in a similar attempt with a Type IR culture. As in mouse passage, reversion by cultural methods was accompanied

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* Rakieten[1119] believed that a peculiar organism obtained from the peritoneal fluid and heart's blood of a mouse after inoculation with a highly virulent Type II Pneumococcus was a pneumococcal variant. It was a Gram-positive bacillus, bile-soluble, agglutinated with Type II serum in a dilution of 1 to 400, and also to a slight extent with Type I serum. The organism was not pathogenic for mice. Rakieten's description of cultural development of the strain from the infected fluids raises doubt as to its true pneumococcal origin.

† Compare Soule's[1308] similar results with *Bacillus subtilis* (1927).

by acquisition of properties of the typical S form. In the experiments, reversion was invariably toward the specific type from which the R form was originally derived.*

REVERSION BY MEANS OF PNEUMOCOCCAL VACCINE

In Griffith's experiments on reversion he introduced a new principle, which later was to effect still more surprising and momentous changes in the biological character of Pneumococcus. He reported that the most certain method of producing reversion was to add to the R culture before subcutaneous injection into the mouse a dose of a heat-killed culture of a virulent strain of the same type. Reversion from R to the S form could occasionally be brought about by the simultaneous inoculation of a virulent culture of another type when the culture had been heated for only a short period to 60°, that is, a Type IIR strain reverted to its original condition when inoculated with a heated, virulent Type I culture. The Type I antigen appeared to lose the power to cause reversion more easily than the Type II antigen, the former becoming inactive after heating to 80°, whereas the latter was still effective after steaming at 100°. Griffith found, moreover, that the antigen of certain Group IV strains appeared to be closely related to that of Type II. Both were equally resistant to heat, and stimulated the reversion of R forms derived from Type II, but failed to bring about the reversion of the RI strain to its S form.

Transformation of Type

More surprising and important was the successful transformation by the method of an R strain derived from one specific type into the S form of the same type as that of the heated culture. The S form of Type I was evolved from the R form of Type II Pneumococcus, and the S form of Type II from an RI organism.

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* Alloway (1932)[8] cited Kelley as having discovered that normal hog serum was rich in these anti-R bodies and could be substituted for anti-R serum in activating the reversion process.

From the RI variant and from the R forms of Type II, were derived the clear, mucinous colonies of Type III. The newly developed Type III strains were of relatively low virulence and frequently remained localized at the site of subcutaneous inoculation. A still wider shift which Griffith effected was that of a Group IVR strain to virulent strains belonging to Types I and II. The injection of large doses of heated cultures of R pneumococci along with small amounts of living R strains never caused a transformation of type and only rarely produced a reversion of the R form of Type II to its S form. Griffith, therefore, along with his success in changing R variants back to the original S forms with accession of virulence and specific type characters, was the first to accomplish a true transformation of one pneumococcal type into another.

To degrade a pneumococcus *in vitro* to a form devoid of its original type characters and then to exalt it to its original condition was an achievement that we had come to expect, but transforming a degenerated or dissociated culture into another form possessing entirely different type characters was a somewhat amazing performance. Even remembering the theories of earlier investigators with their claims of species mutations, and discounting possible errors in their experiments, this discovery had not been anticipated. It was, for the first time, to supply a theoretical explanation for the many baffling problems encountered in the study of the spread and the invasiveness of pneumococci, and of the clinical pathology of pneumococcal infections, not to mention the broader bearing on the many riddles of microbiology.

Neufeld and Levinthal[994] also were able to reproduce Griffith's transformation phenomena, but by another procedure. They first dissociated virulent, type-specific strains by growing the organisms in broth containing sterile animal organs (spleen, heart, kidney, and liver of rabbits). The degraded R variants were then injected subcutaneously into mice with killed S pneumococci. Neufeld and Levinthal thus converted an avirulent Type IR pneumococcus into a virulent Type IS organism, and with the addition of

a killed Type IIS strain obtained a typical IIS pneumococcus. Not all the R variants could be reverted.

Somewhat less success in this respect attended the efforts of Reimann.[1129] The R strains evolved by immune serum-broth cultivation were as a rule irreversible, only one of many strains passing back to the S form of Type I or over to Type III, the reversion depending upon the type of the heated culture used. No transformations to Type II occurred, although in one instance it appeared that a heated IIS culture induced the reversion of the R strain to the Type IS prototype. Reimann obtained positive reversions of typical R forms from pneumococci of Types I or II when he inoculated the R strains subcutaneously into mice with heated S cultures of Types I, II, and III. The living IR culture plus heated Type IIS vaccine gave Type IIS pneumococci; IIR became IS or IIIS, depending upon the type of heated culture used. Both Types IR and IIR, when inoculated with heated cultures of homologous type S forms, frequently reverted to the respective prototypes. These seemingly bizarre biological changes were, therefore, becoming a routine laboratory performance.

Baurhenn's[93] efforts at reversion (1932) were more fruitful than Reimann's. By subculturing R strains with homologous and heterologous vaccines consisting of heat-killed cultures, he changed the R strains into their original S forms and to the S form of a different type. Baurhenn inclined to Griffith's view that all pneumococcal types possess a common basic form. The basic form, under the stimulation of the activating principle, responds by acquiring the properties of the activator. Baurhenn claimed to be the first to have produced transformation within Group X (Group IV) as well as the transformation of a fixed type (I, II, or III) into a specific type of Group X. This feat is, of course, entirely possible, and from what we already know of the phenomena of transformation, there is no reason to doubt that similar changes may occur in the case of all the known types of pneumococci.

Dawson (1928)[299] confirmed and expanded Griffith's observa-

tions. He found that type-specific S pneumococci could be trans-
formed from one specific S type into another specific S type
through the intermediate stage of the R form; that R forms of
pneumococci, derived from any specific S type, might be trans-
formed into S organisms of other specific types by injecting mice
subcutaneously with small amounts of living R strains together
with heated vaccines of heterologous S cultures. The S vaccines
could be heated for fifteen minutes between 60° and 80° and still
remain effective in causing R forms derived from heterologous S
types to revert to the type of the vaccine; S vaccines heated fifteen
minutes at temperatures between 80° and 100° were not active in
causing R variants derived from heterologous S types to revert to
the type of the vaccine; S vaccines heated between 80° and 100°
could cause Type IIR and Type IIIR variants to revert to the
original S type; S vaccines of any type, including Type I, heated
for fifteen minutes at 80° to 100° would no longer cause Type IR
strains to revert to their original S type; S vaccines heated for
periods as long as two hours at 60° were effective in causing R
forms derived from heterologous types to revert to the type of the
vaccine employed. Dawson successfully converted a single-cell R
strain derived from a Type IIIS pneumococcus into a Type IIIS,
a Type IS, and a Group IVS organism. On the other hand, every
attempt to produce transformation of type *in vivo* failed.

TRANSFORMATION BY VACCINE AND ANIMAL INOCULATION

In 1930, Dawson and Sia[305] announced the transformation of a
Type IIR into a Type IIIS pneumococcus. The conditions neces-
sary for the reversal were minimal amounts of the R culture, the
addition of the heated activating culture, incubation for longer
than the conventional period, and the inclusion of small amounts
of anti-R serum and of blood broth. When the activating organ-
isms were heated for fifteen minutes at 100°, they lost their capac-
ity for inducing transformation, although suspensions heated for
four hours at 60° or for fifteen minutes at 80° were still effective.

Filtrates of vigorously growing cultures and of heat-killed suspensions of S organisms were inactive, as also were suspensions of S organisms disrupted by freezing and thawing, with subsequent heating for fifteen minutes at 60°. But when suspensions of S organisms were first killed by heating for fifteen minutes at 60° and then frozen and thawed, they were highly effective. In a more detailed communication, the authors gave the additional information that transformation of type could be induced by the use of small amounts of S vaccine, and that while the transformative process was brought about most readily by employing anti-R serum in the culture medium, it might be accomplished without the presence of the serum.

Transformation of one S form to the S form of a different type without any apparent development of intermediate stages was described by Dawson and Warbasse[306] in 1931. The original culture was a virulent, single-cell isolation of Type II Pneumococcus. One drop of a 10^{-6} dilution of the culture was seeded into a medium containing homologous immune serum together with large quantities of Type III pneumococcal vaccine. The cultures were incubated at 37°, and at the end of forty-eight hours streaked plates showed, in the majority of instances, numerous Type III with some Type IIS colonies. No R colonies were observed. From the experiment Dawson and Warbasse inferred that a type-specific S pneumococcus can be transformed into other type-specific S pneumococci by growth in homologous immune serum in the presence of heterologous vaccine. Although the conditions of the experiment were unfavorable to the development of R forms, the authors thought it was probable that the organism nevertheless passed through this intermediate stage during the transformation.

In 1931, Sia and Dawson[1272] reported that R cultures possessing slight degrees of R stability were most suitable for transformation experiments *in vitro*. The authors also sought a soluble principle in cultures subjected to the action of bacterial enzymes liberated in old broth cultures and during mechanical disruption of

young bacterial cells. Trials with the solutions, with the superna-
tant fluid from an S vaccine, the filtrate from an S vaccine, puri-
fied soluble specific substance, and the filtrates of actively growing
S cultures, all gave negative results.

Alloway (1932)[8] evidently was more successful than his prede-
cessors in obtaining the transformative principle from the pneumo-
coccal cell. With filtered extracts of virulent S strains of Types I
and III he converted a Type IIR strain into S organisms of the
same specific type as that of the cells extracted. The author stated
that the constituents of the extract supplied an activating stimu-
lus of a specific nature in that the R pneumococci acquired the ca-
pacity of elaborating the capsular material peculiar to the organ-
isms extracted.

In the next study (1933), Alloway[9] prepared active and spe-
cific extracts by dissolving S pneumococci in sodium desoxycholate
solution. These cell-free extracts were as potent as the intact cocci
in causing R forms to assume new type-specific characters. With
an extract of Type III Pneumococcus he was able to convert a
Type IIR variant almost regularly and abruptly into the smooth
form of Type III. Alloway then purified the extracts by removing
a considerable amount of inert material by charcoal adsorption
and reprecipitation of the adsorbed extracts with alcohol or ace-
tone. The stimulating principle passed through Berkefeld filters
without loss of strength if the reaction of the solution was alka-
line. The substance was resistant to heating at 60° for thirty min-
utes but was appreciably affected at temperatures of 80° or over.
The purified extracts apparently had suffered no loss of potency
and caused a more prompt transformation than did the original
solutions. An unexplained observation was the fact that in no in-
stance could the transformation be effected without the addition to
the culture-extract mixture of blood serum or of ascitic or pleural
fluid.
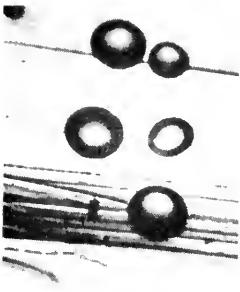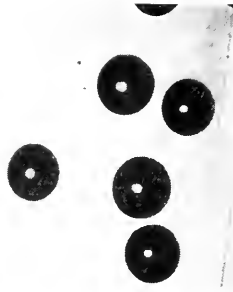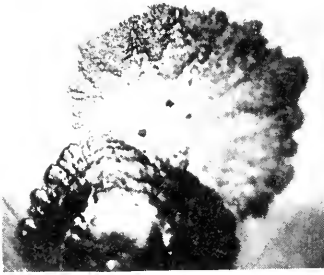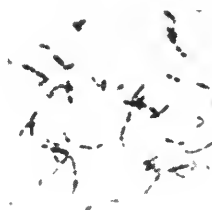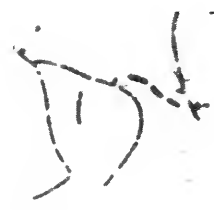
MUCOID, SMOOTH, AND ROUGH PHASES OF PNEUMOCOCCUS

a. Mucoid colonies; twenty-four hours on blood agar. × 32. b. Smooth colonies; twenty-four hours on blood agar. × 32. c. Rough colonies; twenty-four hours on blood agar. × 32. d. Smooth colony with Rough "outbursts"; nine days on blood agar. × 32. e. Mucoid, Smooth, and Rough colonies; sixteen hours on blood agar. The Smooth colony shows a fine granulation or stippling. × 32. f. Mucoid organisms from twenty-four-hour colony on blood agar. Hiss capsule stain. × 1350. g. Smooth organisms from twenty-four-hour colony on blood agar. Gram stain. × 1350. h. Rough organisms from eighteen-hour colony on blood agar. Gram stain. × 1350.

Dawson Classification

A dissociation form, other than the S and R forms, was described in 1934 by Dawson.[303] It appeared to be a mucoid variant of Pneumococcus and was strikingly different from the two main, accepted variants. Dawson intimated that the terminology of bacterial dissociation should be changed to include the M form.

In later communications (1934), Dawson[302-3] gave many more details of the several stages of pneumococcal dissociation. He showed, first, that the change from the typical, virulent form to the degraded variant was not a simple direct S → R conversion, but that the dissociative process consisted of several phases. In this cycle there were three outstanding stages represented by distinct difference in colony appearance and morphology, and here he departed from the orthodox concept of the S and R forms.

At first reading, Dawson's discussion and proposals are a little bewildering. He makes the apparently radical suggestion that the old designations smooth and rough be changed and the term "mucoid" be introduced into the terminology as it applies to Pneumococcus; thus, S would become M; R would become S; and the new form would be R. His revelation of the intricacies of the dissociative phenomena and the proposed change in terminology are apt to cause some confusion in minds accustomed to the accepted order of dissociative nomenclature. But an unprejudiced and painstaking study of the facts and his recommendations serves to dispel some of the doubts raised in a cursory reading of the text. Dawson's contentions were founded on the appearance of a new variant during the cultivation of an R form of Pneumococcus originally derived from a Type IIS culture. When this strain was diluted, thinly streaked on blood-agar plates, and incubated several days at 37°, many of the colonies showed evidence of a variety of secondary growths. The following is taken from Dawson's description of the S → R transformation.

For convenience the evolution of the R variant may be described in several stages although it is emphasized that the process is both gradual

and continuous. In the first stage, which may suitably be termed R^1, the colonies present more or less the general appearance of smooth (old terminology, "rough") colonies but the surface is more coarsely stippled. The constituent organisms are more or less typical pneumococci showing a tendency to staphylococcal grouping and occasional swollen or club forms may be seen. In the second stage, R^2, the colonies present a still rougher appearance and the outline may appear slightly irregular. This irregularity frequently becomes quite pronounced after several days' growth. The bacteria in this stage are much more pleomorphic and are frequently elongated in an extreme lanceolate manner. They still retain Gram's stain. In the third stage, R^3, the surface of the colony becomes exceedingly rough and the margin quite irregular. The contour of such colonies still remains convex but less so than the original S form (old terminology, R form). The organisms constituting such colonies present a bizarre morphological picture. Pointed diphtheroid elements arranged in a fashion suggesting broken twigs may be observed, with scattered long, bizarre, rod forms which are partially Gram-positive and partially Gram-negative. At this stage of development the morphological picture can scarcely be recognized as that of pneumococcus. The fourth stage, R^4, can only be defined with some difficulty. It would appear that the growth is now in a stage of considerable flux and several types of colonies and morphological elements may be produced. Some of the colonies present an appearance similar to that just described while others resemble more closely the pure R form (R^n).

The morphology of the organisms in the R^4 stage is difficult to describe because of their extreme pleomorphism. In addition to coarse and irregular coccal forms there may appear elongated Gram-negative rod-like structures exhibiting irregular Gram-positive areas. A great variety of other morphological elements may also be present.

Dawson described the R → S change in which intermediate forms of the type seen in the S → R change were not observed, and then gave a detailed description of the biological characters of this new variant. From the description a few of the more important data may be selected. The organism was bile-soluble, of low virulence for mice, agglutinated in normal saline solution, and failed to elaborate soluble specific substance. The variant was not peculiar to the

Type IIR strain, and single organisms of one individual strain also possessed the capacity to dissociate into the new form.

Dawson then pointed out certain discrepancies in the characteristic features of the S and R forms as described by Griffith for Pneumococcus and those described by Arkwright for the colon-typhoid-dysentery group, and which have been accepted by the majority of bacteriologists as the chief distinguishing features of the smooth and rough forms of many bacterial species. He further drew attention to the fact that certain attributes of Arkwright's S and R forms do not appear in Pneumococcus while other new distinctions did not have a place in Arkwright's original descriptions. Dawson has portrayed these differences in terminology in a diagram which, although as yet unpublished, was kindly loaned to the authors.

	COLON, TYPHOID, DYSENTERY		PNEUMOCOCCUS	
	ARKWRIGHT	OTHER OBSERVERS	GRIFFITHS	DAWSON
1	NOT DESCRIBED	M	S	M
2	S	S	R	S
3	R	R	NOT DESCRIBED	R

Courtesy of Dr. M. A. Dawson

RELATIONSHIPS OF MUCOID, SMOOTH, AND ROUGH COLONIES OF BACILLI OF THE COLON-TYPHOID-DYSENTERY GROUP AND PNEUMOCOCCUS

When Dawson compared the salient characters of the three pneumococcal variants (S, R, and the new M form) with the mucoid, smooth, and rough forms of members of the colon-typhoid-dysentery group and the smooth and the two rough forms of the Friedländer bacillus, the inconsistency in the use of the terms smooth and rough became convincingly apparent. On a basis of colony appearance, morphology, growth in plain broth, stability in salt solution, and of virulence and type-specificity, the smooth form of Pneumococcus and of Friedländer's bacillus conforms to the mucoid form of members of the colon-typhoid-dysentery group; the rough form of Pneumococcus and the R_1 form of Friedländer are similar to the smooth form of bacilli of the enteric group; while Dawson's new variant and Julianelle's R_2 form of the Friedländer bacillus agree with the rough form of the colon-typhoid-dysentery bacilli.

In order, therefore, to bring these terms in agreement, to conform—with an addition—to the designations of Arkwright, and to establish a uniform and logical terminology for the dissociants of all bacterial species, Dawson would change the terms now used for the variants of pneumococci as follows: Mucoid or M would replace the present smooth or S; smooth would be substituted for the former rough (R_1 form of Friedländer bacilli); while rough or R would be applied to the new variant described by Dawson and the R_2 form of Friedländer's bacillus.

There is no doubt that such a reversal of the accepted terms would cause confusion and meet with opposition. It cannot be denied that this change would be especially disturbing to the present correlation between the classification of dissociation forms and immunological behavior, but that does not necessarily preclude the possibility of a new and perhaps a deeper insight into the parallelism between the phenomena of variation and antigenic specificity. This proposed change recalls the confusion that followed the revision of the designations of blood groups, but that change has not only been endured but the new terms are now generally accepted as

useful and logical. There is much to be said both for and against Dawson's proposal and so it may be permissible to turn to one who speaks with authority on this important subject of bacterial dissociation. Hadley's opinion expressed in a letter written in 1933 to Dawson was in part:*

Making a decision regarding the proper course to pursue in changing the nomenclature now employed for designating the phases of the pneumococcus, in favor of the symbolization which your studies thus far seem unquestionably to justify, might easily depend on how fully an investigator has in mind the details of dissociative variation as a phenomenon observable in all bacterial species, and how clearly he can perceive the parallel trends in such variations,—as opposed to a limited outlook on the one species in which he may be especially interested.

If bacteriology were limited to the study of a few species, or to the Pneumococcus, it would make little difference what the observed phases were called, because no generalizations would be involved, and the phase symbols would possess no significance for bacteriology as a whole. A, B and C, or X, Y and Z would serve the purpose. . . .

The desirability of adjusting the difficulty in the Pneumococcus situation, and of doing it without delay, is the more to be recommended in view of the increasingly wide recognition that the same or analogous phases exist in numerous other species. The facts are now becoming so extensive and well grounded that they are offering, for the first time in the history of bacteriology, a basis for the formulation of general laws; and for making possible a certain kind of "predictability," as I have perhaps already demonstrated to you. To this extent pure bacteriology is beginning to take on the aspects of a real science—a compliment which (to my mind) it has scarcely been appropriate to offer in the past.

To facilitate this highly gratifying trend it stands to reason that all who work with the problems of variation should keep in mind the dual significance of their results, and make possible a correlation of their own results with those of others; also to make quick and decisive corrections when such are clearly in order. To label as a smooth a Pneumococcus phase that is demonstrated to be a mucoid, or to label as a rough a phase that is clearly a smooth, may do little harm to those

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* The authors appreciate the courtesy of Doctors Hadley and Dawson in granting permission to include portions of this letter here.

whose work lies chiefly in this species. But such a continued policy can only render increasingly difficult important comparisons with other species, and work havoc with the interests of those who are seriously attempting to discern some law and order in the affairs of the bacteria. Further advance in this direction can take place, according to my view. only if bacteriologists become sufficiently keen to recognize the true nature of the phases they employ, and sufficiently independent to "call a spade a spade," whenever recognized as such, regardless of politics, tradition or social etiquette. . . .

It might also be in the back of your mind that the splendid work of some of your associates on the chemical aspects of dissociation would suffer from any change in terminology made at this late date. I am absolutely convinced to the contrary. In reality I believe that the incentive to extensions of their results to many other bacterial species would be a direct and immediate outcome, through establishing a recognition of the most appropriate culture phase to be employed in such studies. . . .

It is therefore my opinion that a frank recognition of the present incongruities of the situation will not detract from, but facilitate in wide measure, researches in the important field opened up years ago by Drs. Avery, Dochez, Heidelberger and their collaborators.

Dawson believed that before making such a radical change in the accepted terminology of pneumococcal variants it would be well to ascertain if similar variants could be demonstrated in *Streptococcus haemolyticus*. From the latest study by Dawson,[303] it would seem that he succeeded in dissociating that organism into three main variants, which in their manner of colony formation and in morphology correspond closely with the three main variants of Pneumococcus. The mucoid and smooth forms appeared and, by cultivation of the streptococci on blood agar and by repeatedly picking and transplanting material from the roughest marginal areas, Dawson was able to develop the extremely rough type of colony which he had obtained with pneumococci, representing the R variant.

As Dawson said, "evidence is rapidly accumulating to show that the phenomenon of bacterial variation in a wide variety of bac-

terial species fits into a more or less orderly pattern." This pattern, besides fitting bacilli of the colon-typhoid-dysentery group, the types of *B. friedländeri*, and probably the streptococci, would bring order in the arrangement of the many variants of pneumococci that have been described under a wide diversity of terms. Thus, the modifications A, B, and C of Schnitzer and Berger, Blake and Trask's intermediates Type I a, b, c, d, and e, Wadsworth and Sickles' atypical strains, Reimann's daughter-colony variants, the "wall" type of Buerger, the *Flätterformen* of Grumbach, possibly the P–C or phantom colonies and the smooth N and the smooth V types of Eaton, the variants of Kimura, Sukneff, and Meyer, the atypical rough forms from budding colonies reported by Paul, the SU and RK dissociants of Klumpen, and of course the R and S forms of Griffith, and the new variant of Dawson might conceivably be arranged in accordance with the general pattern and would all either fall into the chief places designated by Dawson's M, S, and R or into the spaces between these predominating forms.

The scheme of Dawson, therefore, revolutionary as it may seem, merits further consideration and should be subjected to additional experimental trial before it is rejected or finally accepted.

These discoveries concerning the variability of Pneumococcus are full of new meaning to the bacteriologist, biochemist, immunologist, and particularly to the physiologist. They prove that Pneumococcus has the potential ability to synthesize simple sugars into diverse, complex, and highly individual polysaccharides. When the conditions of the surroundings are entirely favorable, this metabolic process operates uniformly. The end products are always of the same molecular composition and configuration, and are highly distinctive of a given serological and biochemical type. When, however, the forces of the environment are inimical, the function of carbohydrate synthesis is retarded, the cell produces less and less of the distinguishing capsular polysaccharide, and the cocci lose their capsule, virulence, and strict racial identity. If the

unfavorable conditions continue, this particular metabolic activity ceases or is suppressed and the organism degenerates into a harmless coccus, devoid of any specialized earmarks—a sort of bacterial maverick. If the exposure to these untoward conditions is sufficiently protracted, the function is apparently permanently lost, but if the exposure ceases before this stage is reached, the cell retains the latent power to elaborate its original, individual capsular carbohydrate, and all that is needed to revive this power is the restoration of a satisfactory environment—either in culture or in an animal—or else the activation that comes from an encounter with immune bodies specific for its own degraded form. Living under such conditions the type-less coccus gradually returns to its former distinctive state.

These discoveries, moreover, have disclosed another and astonishing activity of the organism. When stimulated by some unknown constituent of fully functioning pneumococcal cells, this latent metabolic function of the degenerated coccus develops a new property, and instead of building up capsular carbohydrates of the former kind, the degraded cell now synthesizes polysaccharides of the same chemical constitution and specific type as those of the strains supplying the activating stimulus. The once degraded organism becomes then a virulent pneumococcus, but with all the specialized characters of its foster strain. Having lost its original features it regains a new type identity.

The cycle of degradation, regeneration, and type transformation presents so many fascinating phases that one is strongly tempted to speculate on the various factors concerned in this extraordinary evolution. The basic ability to elaborate these various specific capsular carbohydrates is always ready to respond to appropriate stimulation unless the cells have gone too far down the path of degradation, and is evidently common to all pneumococci. The direction which the transformation takes is determined wholly by the nature of the stimulus, and it is the identity of this factor which still remains unrevealed to us. It apparently exists only in

cells exercising all their special functions, and seems to be a normal constituent of the cell and not a product of katabolic processes.

Whether such transformations ever take place in the animal body, in health or in disease, and if they do what causes bring them about, together with the yet broader problems of the origin of various types and the influences which established their different biological identities, are all questions that are attracting investigators in this branch of science. Whether this fundamental function of Pneumococcus can be so perverted as to bring about the transmutation of this organism into one of a different species is a problem which has been attacked in a more general way.

Transmutation of Species

The mutability of members of the bacterial tribe *Streptococcaceae* has long been a moot question. From time to time there have appeared reports of the change of a pneumococcus into a streptococcus, and even of a swing through the whole cycle from virulent Pneumococcus to *Streptococcus viridans* to *Streptococcus haemolyticus* and back to Pneumococcus. But, in these later days of refined bacteriological and immunological technique one has been inclined to look somewhat askance at such claims. The idea has, however, persisted, and what was looked upon as a mere notion is now becoming so much more than a hypothesis that there are those who would accept this metamorphosis as an accomplished fact.

There is no call to recite at any length the accounts of the early experiments. Some were based on crude, faulty methods which always raise doubts as to the purity of the cultures the pioneers studied. Disregarding claims resting solely upon morphological or cultural phenomena, it is better to confine the discussion to reports, with a few exceptions of historical interest, that have been published since the development of modern bacteriological and serological technique. In 1891, Kruse and Pansini,[763] by transplanting forty-six strains of pneumococci on media unfavorable to growth, developed eighty-four varieties that exhibited differences

in character all the way from typical *Diplococcus lanceolatus* to *Streptococcus pyogenes*. The authors stated that the relation of pneumococci to streptococci was clearly evident, and that the origin of these bacterial species was a single, probably saprophytic, streptococcal form.

There the matter rested until 1907, when Buerger and Ryttenberg[169] described an organism isolated from a case of puerperal pneumococcemia which, although originally failing to ferment inulin and exhibiting streptococcal characters, developed into a typical pneumococcus after animal passage. The observation led the authors to study a number of cultures isolated from human exudates and blood, and with these strains they observed characters typical of streptococci which, however, gave way to pneumococcal characters after propagation in mice. Buerger and Ryttenberg concluded:

> The tendency of pneumococci of the streptococcus cultural type as well as those which have been converted to the normal variety, seems to be toward a gradual degeneration which manifests itself in the assumption of permanent streptococcic features. Such organisms can then no longer be differentiated from streptococci.

In 1909, Rosenow[1162] made the statement that strains of *Streptococcus viridans* isolated chiefly from the blood in cases of subacute endocarditis and obtained also from the throat and other sources might by animal passage take on the properties of typical pneumococci, and hence designated them as "modified pneumococci." Rosenow also claimed that during a study of autolysis of pneumococci in salt solution and of the effect of sodium oleate and bile on virulent pneumococci he had observed transformation of the strains into hemolytic streptococci. The statement appears to be conservative when compared to Rosenow's[1170] description in 1914 of the various transmutations accomplished within the Streptococcus-Pneumococcus group. He told of converting by cultural methods twenty-one strains originally isolated as hemolytic strep-

tococci from cases of erysipelas, scarlet fever, puerperal sepsis, arthritis, and tonsillitis, as well as from cows' milk, into *Streptococcus viridans;* of changing three similar strains into *S. viridans* and typical pneumococci, and one into *Streptococcus mucosus* as well. Seventeen strains isolated as *S. viridans,* chiefly from the blood and tonsils in cases of chronic infectious endocarditis, and two strains from cows' milk were converted into pneumococci while two of the strains became *S. mucosus.* Ten of the *viridans* cultures were made to take on the cultural and morphological characters of hemolytic streptococci, in two of which the pathogenic powers were shown to be those of hemolytic streptococci, while one strain was converted into a hemolytic streptococcus, into *S. viridans,* and then into a pneumococcus.

Rosenow claimed to have converted into hemolytic streptococci eleven strains isolated as pneumococci from sputum, blood, and the lung in pneumonia and from human empyema fluids and Cole's Type I and II strains, while seven cultures took on the features of *S. viridans.* The streptococci derived by animal passage from three of the pneumococcal strains were alleged to acquire all the essential features of the streptococci of rheumatism, and two organisms were said to have been converted into hemolytic streptococci, the streptococci of rheumatism, *S. viridans,* and back again into Pneumococcus.

Rosenow further alleged that the transformation of some of these strains, checked in a few instances by single-cell isolations, was found to be complete by every test known. The tests included the study of morphological features, the demonstration of capsules, and observations on fermentative powers, solubility in bile and in saline solution, the behavior toward the respective broth-culture filtrates (Marmorek's test), the specific immunological response as manifested by the appearance of opsonin and agglutinin in antistreptococcic and antipneumococcic serum, and the more or less specific pathogenic powers of the various organisms.

In summary Rosenow wrote:

The changes observed have frequently the characteristics of true mu-
tations because they appear suddenly, under conditions more or less ob-
scure and because the newly acquired properties persist unless the or-
ganisms are again placed under special conditions. A pre-mutational
stage seems to be necessary because the same strain will not yield mu-
tants when placed under what seem to be identical conditions at differ-
ent times. The underlying conditions which tend most to call forth
changes are, first, favorable conditions for luxuriant growth and then
unfavorable conditions—under stress and strain. This seems to call
forth new or latent energies which were previously not manifest and
which now have gained the ascendency and tend to persist. This may
hold true *in vivo* also. This fact makes it difficult to obtain mutations
outside of the body with highly virulent strains, because they die before
there is opportunity for the organisms to adjust themselves to the new
conditions. It explains why injection into cavities makes for greater
changes than intravenous injections of moderately virulent organisms.
Apparent mutations in animals have been observed almost exclusively in
closed cavities, such as joints and pericardium, and here mostly when
the tissues of the host were gradually getting the upper hand and the
organisms were being destroyed. The mutations *in vitro* may be spoken
of as "retrogressive" and those in animals as "progressive" because evi-
dences of a vigorous vegetative life are diminished whereas in the latter
they are usually increased.

The results and conclusions of Rosenow have been transcribed in
some detail because they represented such a wide departure from
established belief. The announcement was greeted with much skep-
ticism. Such sudden and wide shifts from one to another suppos-
edly fixed species appeared to violate biological laws, and it seemed
that some artifact must have been responsible for the remarkable
transformations. Nowhere in the literature, with the exceptions to
be described, have references been found which duplicate or sub-
stantiate Rosenow's results.

Wolff (1923),[1534] in a long theoretical discussion of pneumococ-
cal mutation, suggested that the members of the large tribe *Strep-
tococcaceae*, from pure saprophytes to true parasites, in spite of

all differences, were really linked together. He claimed to have obtained mutations by gradual adaptations of the organisms to the host. The attempts met with many failures which were explained by saying that if the organism was too weak it died in the host, and if too virulent it killed the host before any accommodation had taken place. Wolff asserted, however, that in three cases he had transformed *Streptococcus viridans* from *endocarditis lenta* into Pneumococcus. The organism became bile-soluble, optochin-sensitive, developed a capsule, fermented inulin, and was lethal for mice. Evidence of bacterial mutations of any kind coming solely from *in vivo* experiments is to be weighed with caution.

Neufeld[979] in a discussion already cited on microbic variability, recalled an observation he had made ten years previously on the original "Pneumococcus I" of Neufeld and Haendel, which had been preserved by drying and storage in a dessicator. One mouse inoculated with the culture produced a strain growing in chains, insoluble in bile, but virulent for mice, and with all typical streptococcal properties. At first Neufeld thought he had made a mistake in the material he injected, but a similar experience of Schiemann's convinced him that a mutation had actually taken place. Coming from anyone less eminent than Neufeld, this single, isolated observation would be disregarded.

In the following year, Morgenroth, Schnitzer, and Berger[929] announced that by special methods they had been able with regularity to transform pneumococci into streptococci.* Their medium contained dead yeast cells or animal charcoal which had adsorbed optochin. The altered strains became insoluble in sodium taurocholate, were avirulent for mice, and were resistant to optochin. Modification A represented the first stage in the transmutation. The organisms retained the majority of their pneumococcal characters, but were more resistant to optochin and more sensitive to

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* Stanka[1312] called attention to the fact that these authors had omitted mention of similar work published by Elschnig and Ulbrich, and by Kraupa from the German Eye Clinic at Prague.

rivanol than were cocci of the original stock. In Modification B, the colonies, made up of A after growing in optochin, resembled those of *S. viridans*. The cultures contained long chains of round cocci, which were bile-insoluble and were very resistant to the pneumococcidal action of optochin. Modification C developed after further growth on artificial media or in animals, and occasionally after growth in an optochin medium. The C variants corresponded to *Streptococcus haemolyticus*, they produced more or less hemolysis on blood agar, were bile-insoluble and optochin-fast, but sensitive to rivanol. The progressive changes did not always take place or follow the A-B-C sequence. In twenty-nine experiments with fifteen strains, twenty-two trials produced modifications A and B, and of these strains seven were transformed into modification C.

Berger and Englemann[100] continued similar mutation experiments and alleged to have demonstrated Modification A in five specimens of sputum and one of pleural exudate obtained from pneumonia patients before the disappearance of fever. The strains were then converted into Modification B by allowing a fairly high concentration of optochin to act upon them. Berger and Englemann also claimed that the complete transformation could take place in the human organism. To support the claim the authors described the development of glistening Type III colonies along with a few strongly hemolytic streptococcal colonies on a blood-agar plate upon which pneumonic sputum had been spread. The organism, after the first mouse passage and three culture generations, developed into a green streptococcus; after a second direct mouse passage both pneumococci and hemolytic streptococci appeared, the latter partly reverting to Pneumococcus after two culture generations. The original hemolytic streptococci after three culture generations became green streptococci and after four culture generations reverted to pneumococci. This cycle, like Rosenow's, seems almost too rapid and direct to be credible.

In another communication, Berger and Jakob (1925)[102] returned

to earlier experiments on the development of B and C modifica-
tions. During animal passage of short duration, the changes were
less marked, since the authors reported only a transient loss of
virulence. Berger and Englemann[101] in the next year reported the
mutation of a strain of Type III Pneumococcus through the in-
termediary A modification to a green streptococcus. As in their
former experiments, the agents necessary for the transformation
were dry yeast-broth and serum-broth containing one five-thou-
sandth part optochin. Wirth[1523] believed that *Streptococcus mu-
cosus* represented a mutation from Pneumococcus, but he failed in
his attempts to prove it.

In yet another paper Berger with Silberstein[103] described the
inulin-fermentative power of the variants. The results are difficult
to understand. Of ten strains of pneumococci, four showed merely
a reddening of the inulin medium without coagulation, while two
failed to display any action on inulin. The authors then classed
the latter strains when tested with optochin with Modification A.
Of the cultures of Modification B, obtained from pneumococci, but
otherwise behaving as green streptococci, two retained the ability
to ferment the carbohydrate. The strains were comparable in their
behavior toward inulin to some thirty *viridans* strains. Of the lat-
ter, five exhibited a marked action on inulin, and four others gave
slightly positive reactions.

Reimann,[1127] repeating the experiments of Morgenroth, Schnit-
zer, and Berger, claimed, however, that the R cultures so derived
were still pneumococci, since the strains were bile-soluble and
autolyzed with greater readiness than did streptococci. The immu-
nological reactions of the variant pneumococci derived by Morgen-
roth's method, moreover, were identical with those of R pneumo-
cocci derived by various other means. When one considers the
atypical action of the Berger strains on inulin and the author's
omission of serological tests, one is inclined to accept Reimann's
interpretation as the correct one.

Heim and Schlirf,[633] likewise, were unable to verify the work of

Morgenroth and his collaborators, yet Silberstein,[1286] who quoted these authors, by the aid of optochin *in vitro*, claimed to have experienced no difficulty in carrying a Group IV pneumococcus through the successive stages of Modification B (green Streptococcus) to Modification C (virulent hemolytic Streptococcus) and then from this form to a Type I pneumococcus of low virulence. Paul[1070] was another to join the newer school which believed that the gap between pneumococci and streptococci could be bridged by these methods. He produced bile-insoluble dissociants and to him they appeared to be indistinguishable from certain strains of *Streptococcus viridans.*

Görander (1930)[542] also stated that he had transmuted cultures of *Streptococcus viridans* into bacterial forms that in every respect were identical with the type-specific pneumococci of human origin, except that the strains were not agglutinated by antipneumococcic serum. The defect would seem to be a vital one. The cultural changes were accomplished by repeated cultivation on blood agar and by short mouse passages. According to Görander, after the third short (four-hour) mouse passage, hemolytic streptococci appeared. Following five twenty-four hour incubation periods in mice, the organisms resembled pneumococci. The variants had capsules, were soluble in sodium taurocholate, and were moderately virulent for mice. The pneumococci so obtained, after repeated growth of this passage culture in artificial media (alternating blood agar and broth), were retransformed "into a bacterium of perfect *Streptococcus viridans* type."

Görander claimed further to have transformed *Streptococcus viridans* and Type I and Type II pneumococci into forms which he considered to be their original state, "since they were absolutely equal culturally, biologically and serologically in all respects." The homologous antiserum agglutinated both strains, and "the bacteria absorbed not only their homologous but also heterologous agglutinins from both sera." Görander's further conclusions were so heterodox that they are quoted here:

Finally single cell cultures originating in their time from a single cell of a pneumococcus have been examined with regard to the degree of dissimilarity which such cultures can eventually show. These experiments gave the result that two pneumococcus cultures, obtained from the same cell, can show much greater dissimilarities than two cultures obtained one from a typical *Streptococcus viridans* and the other from a typical *Streptococcus lanceolatus.* . . . Thus *Streptococcus viridans* and *Pneumococcus lanceolatus* seem to be different forms of the same bacterium, and the specific agglutinability, which *Pneumococcus lanceolatus* shows when grown from the human body and which has been taken as a base for the so-called type classification, is only an occasional character.

Summary

Virulent pneumococci of all the known serological types, upon encountering unfavorable physical, nutritional, or other biochemical conditions during growth or storage, undergo marked changes in virulence, in ability to elaborate capsules, in colony development, and in their immunological characters. In studies on the dissociation phenomena displayed by pneumococci, a great variety of aberrant coccal forms have been observed which are intermediate between the typical, virulent form and the thoroughly degraded, atypical form. So many variants with such a diversity of biological characters have been described and so many designations have been given to the intermediate forms, that it is difficult to gain a clear conception of the significance of the many phases of pneumococcal dissociation. In order to bring order out of this chaos and to make the nomenclature applied to pneumococci uniform with that employed in naming the variants occurring in the case of other bacterial species, it has been proposed to change the terminology now in use. Mucoid or M would replace the present smooth or S; smooth would be substituted for rough; while rough or R would apply to a recently discovered variant. Whatever the fate of the proposal, the alterations in character which may be induced in pneumococci by appropriate means constitute one of the most important features in the biology of the species.

During the dissociative process antigenic action may vary from one of strict type-specificity to one merely of the broader species-specificity. Degraded forms may, if the degenerative process has not been complete, regain all their original morphological, cultural, and immunological characters. Regeneration can be accomplished by rejuvenating the strain by passage through a susceptible animal, by cultivation in media containing an antiserum produced by immunization with the degraded forms, or through the stimulus afforded by heat-killed virulent cultures of an homologous type. Degraded variants, moreover, can by the action of devitalized, virulent pneumococci, actually be transformed into pneumococci of types entirely different from those from which the variants were derived and identical with those of the cultures stimulating transformation. The nature of this transformative or mutative principle is still unknown, but it is probable that it is a constituent of the pneumococcal cell and not an extracellular product of its metabolism.

The broader transmutation of Pneumococcus into Streptococcus and of Streptococcus into Pneumococcus has been advanced as a biological possibility. Experiments have been described in which it was alleged that this transmutation took place. Not only has it been claimed that both virulent and degraded pneumococci were converted into avirulent *Streptococcus viridans*, but the organisms were said to have become virulent hemolytic streptococci, while the streptococcal forms have been further changed into pneumococci. Such radical departures from established theory require the closest scrutiny of the evidence advanced and of the accumulation of new and confirmatory facts before they can be accepted.

PATHOGENICITY OF PNEUMOCOCCUS: EXPERIMENTAL ANIMALS

The ability of Pneumococcus to infect animals of different species under natural circumstances or when experimentally introduced by diverse routes into the animal body; the morbid manifestations following pneumococcal infection in animals; virulence and other factors influencing infectivity of pneumococci.

Pₙᴇᴜᴍᴏᴄᴏᴄᴄᴜꜱ is incapable of producing disease in animals of some species, while individual creatures exhibit different degrees of resistance to pneumococcal invasion. Variations in susceptibility or in resistance may be conditioned by special differences in anatomic structure, in genetic heritage, or in physiological function, while individual differences may be due to both intrinsic and extrinsic factors affecting the physical state of the animal before or at the time of inoculation.

Susceptibility of the Animal Host

THE RABBIT

Rabbits are prone to develop spontaneous pneumococcal infection of the respiratory tract, contracting the disease from similarly affected guinea pigs or from other rabbits.* Recovery from the infection may be followed by the carrier state during which the animal may serve as a potential source of infection to other stock animals, while the presence of pneumococci in the nasopharynx may constitute a confusing factor when the animal is used for experimental pneumococcal infection.

Next to the mouse, the rabbit is the most susceptible of labora-

* This is not an uncommon laboratory experience and among the references at hand is that of Sanderson,[1218] who wrote of the spontaneous death of a rabbit from Pneumococcus following a supposedly air-borne infection in the laboratory animal room.

tory animals to pneumococcal infection. Susceptibility is greatest
for strains of Type I pneumococci, less for Type II organisms, and
still less for those of Type III. It should be remembered that later
studies have shown that while many strains of Type III pneumo-
cocci are avirulent for rabbits, there are others of the same type
that are highly pathogenic.

It will be recalled that in the early days of bacteriology, Pas-
teur,[1065-6] Sternberg,[1316-8] Vulpian,[1453] and Claxton[237] produced a
fatal septicemia in rabbits by the subcutaneous injection of human
saliva containing, as we now know, pneumococci. With the excep-
tion of the intact skin, rabbit tissues present no barriers to the in-
vasion of virulent pneumococci; the organisms, no matter by
which avenue introduced, soon reach the blood stream and, when in
sufficient numbers, cause the death of the animal.

Bacteriemia is the predominant manifestation and pneumonia
develops only when the organisms are implanted in the lung by way
of the respiratory tract. The weakness of the rabbits' defense
against subcutaneous, intraperitoneal, or intravenous pneumococ-
cal inoculation is due, in part, to the comparative inability of the
leucocytes of the animal to engulf the invading cocci (Tongs,
1922[1416]). Even when injected into the cisternal cavity of the
brain, as reported by Stewart (1927),[1322] septicemia rather than
meningitis ensues. As a rule, therefore, after artificial inoculation
with pneumococci, infection, unless it be by the intradermal or in-
tratracheal route, tends to become systemic and not localized.

Subcutaneous inoculation. Neufeld (1901)[973] spoke of a pro-
gressive inflammation following the injection of pneumococci into
the subcutaneous tissues of the ear of the rabbit. When death did
not follow, necrosis was observed, and the infection appeared to
Neufeld to be similar to erysipelas, although Fraenkel, who had
previously observed the same effect, had not so considered it.
Cooper[276] found that the mucous membrane of the buccal surface
of the rabbit's cheek was susceptible to infection, but the inocula-

tion in reality was a subcutaneous one, since it was necessary to injure the membrane by scratching it before infection took place.

Intravenous inoculation. The rapid and frequently fatal infection resulting from the injection of pneumococci into the circulatory system has often been utilized for testing the specific resistance of rabbits after various immunizing treatments. Among the many references, there might be mentioned the observations of Tillett (1927)[1403-4] who, in attempts to stimulate the production of immune bodies, discovered differences in the pathogenicity of certain Type III strains for man and for rabbits. Some of the strains isolated from human sources, despite the possession of large capsules and high virulence for mice, exhibited low virulence for rabbits. The intravenous injection of the Type III strains avirulent for the species produced a non-fatal bacteriemia which, however, in its course differed from that caused by non-encapsulated, rough forms of pneumococci.

Intradermal inoculation. In 1928, Goodner[525-6] described the acute and often fatal infection developing in the rabbit following the introduction of Type I Pneumococcus into the skin. After the injection of a small quantity of a broth culture of the organism into the skin at the midline of the abdomen, within eight to twelve hours there appears a local lesion, consisting of a swollen, edematous area which may spread until the whole midabdominal region is involved. The development of the lesion is accompanied by an abrupt rise in temperature and invasion by the cocci of the blood stream. A varying proportion of the animals so treated spontaneously recover and, as a result of the infection, may become temporarily immune. Goodner pointed to the analogy between the nature of the localized and subsequent systemic infection arising after intradermal inoculation of the rabbit with Pneumococcus and that of lobar pneumonia in man. Since it does not come within the province of the present volume to discuss, except in a cursory way, the pathological processes caused by pneumococcal infection, the reader

is referred to the original communications of Goodner,[525-7, 541] Rhoades and Goodner,[1136] and others who have described the intimate details of the phenomena.

Kolmer and Rule[748] employed the intradermal method to test the resistance of rabbits induced by previous immunization with pneumococci; Goodner, Dubos and Avery,[536] and Goodner and Dubos,[535] for studying the effect of the polysaccharide-splitting bacterial enzyme in infection with Type III Pneumococcus; while Goodner,[527] Watson and Cooper,[1492] Powell, Jamieson, Bailey and Hyde,[1105] Sabin,[1208] Gelarie and Sabin,[510] and Curphey and Baruch[293] applied the Goodner technique in determining the immunizing action of specific immune serum and other agents.

Inoculation by way of the respiratory tract. Tchistovitch (1890)[1381] was apparently the first to study the effect of Pneumococcus when introduced into the trachea of the rabbit. The diplococci caused only a feeble, local inflammatory reaction with little phagocytosis. In 1915, Kline and Winternitz[728] described the conditions necessary to produce lobar pneumonia in rabbits. The catheter must be inserted as deeply as possible into a bronchus, and the culture fluid must be injected with considerable force in order that the organisms may be introduced into the alveoli.

Permar[1082] has described in detail the manifestations appearing after the intratracheal injections of cultures of Type I Pneumococcus. He concluded that experimental pneumonia in the rabbit begins as an acute inflammatory reaction. The severity of the reaction increases from the trachea and bronchioles and is greatest in the bud-like alveoli arising from them, in the alveolar ducts, atrea, and alveoli. The process begins in the bronchus and invades other tissues by peripheral extension leading to coalescence. Acute interstitial pneumonia in the rabbit develops early as a result of acute lymphangitis arising in the peripheral lymphatics; then the process extends to both pleura and hilum. Permar suggested that septicemia might arise as the result of the direct involvement of the vascular walls, or it might possibly be due to the passage of organ-

isms through the nodes at the hilum and thence through the thoracic duct into the circulatory system. The author believed that the process was, in its essentials, comparable to that occurring in spontaneous pneumonia in human beings, the chief difference being a more intense interstitial involvement in the experimental disease.

Through the insufflation of the lung with cultures of pneumococci of Types I, II, and III, and of Group IV, Gaskell (1925)[500] reported that organisms of Group IV possessed a greater pathogenicity for the rabbit than those of Type I which, in turn, were more invasive than those of Type II, while the Type III culture employed was the least virulent of all.

Stuppy and Falk (1931)[1352] found that intrabronchial insufflation of rabbits with cultures of pneumococci of uniformly high virulence gave rise to bronchopneumonia which, with septicemia and a generalized distribution of cocci in the lungs, usually caused the death of the animal in two to five days. In some animals there was acute inflammation of the interstitial tissue of the lung, with perivascular and peribronchial lymphangitis. Suppurative bronchitis and pleuritis were only occasionally seen. On the whole, the pulmonary lesions induced in rabbits by strains of Type I, II, and III pneumococci of the same virulence were quite similar, while individual virulence rather than the serological type of the culture employed appeared to be the important factor in establishing infection. In a study of the effects of the inhalation of pneumococci, Stillman[1336] observed that, following the spraying of rabbits with cultures of virulent Type III cultures, the organisms tended to remain in the lungs for a considerable period of time without invading the blood stream. When once the organisms had reached the blood, a fatal septicemia resulted. The course of events was in contrast to that ensuing after the similar administration of Type I and II strains. Organisms of Types I and II frequently entered the circulation, but in such instances, only a relatively small number of the animals died.

Another organ of the rabbit possessing little or no resistance to

infection with Pneumococcus is the eye. Tchistovitch (1890),[1381] on introducing the organism into the anterior chamber, found that the aqueous humor, instead of being antagonistic to the cocci, served as a medium for their development. According to Neufeld and Schnitzer, injection of virulent strains of pneumococci under the conjunctiva or cornea leads to a severe infection of the eye, which is followed by systemic infection. The same result attended a similar injection of mouse blood containing pneumococci (Ginsberg and Kaufmann, 1913[517]).

Chilling and wetting, age, breed, weight, and diet as factors influencing susceptibility. The great variability in the behavior of rabbits toward pneumococcal infection, whether naturally or artificially acquired, may be due to either or both internal or external conditions. Among external causes Kline and Winternitz (1915)[728] studied the influence of cold, alcohol, ether, and bromine on rabbits infected intrabronchially with Pneumococcus. The agents appeared to predispose the animal to the development of bronchitis and even bronchopneumonia, but the results were not conclusive. In experiments in which rabbits were given intrabronchial inoculations of virulent pneumococci, Stuppy and Falk[1352] found that wetting and chilling the animals failed to lower resistance to invasion of the injected pneumococci, but exposure to cold appeared to render the test animals more susceptible to spontaneous infection.

Freund[484] observed the difference between the reactions of young and adult rabbits to intradermal inoculation with virulent pneumococci—confirming the susceptibility of immature individuals earlier reported by Kruse and Pansini.[763] In adult rabbits injected with virulent pneumococci extensive inflammation developed at the site of infection, but bacteriemia and death occurred in relatively few of the animals. Younger animals failed to develop such an extensive inflammatory process and succumbed to bacteriemia. The ability to respond to inoculation with an energetic, local reaction constitutes a barrier which apparently develops with growth of the animal.

In an investigation of the physiological variables responsible for the lack of uniformity in the behavior of rabbits to intradermal inoculation with Pneumococcus, Goodner[532] concluded that, since normal rabbits lacked any form of specific antipneumococcal antibodies, resistance to intradermal inoculation in combination with passive immunization with specific immune serum was determined by the physiological condition favorable to the utilization of passively conferred specific antibodies, which in turn depended upon the weight and white blood-cell count of the rabbits. Animals, therefore, that are physiologically mature possess an advantage over animals less mature and with a lower cell activity.

Another factor affecting the resistance of rabbits to infection is vitamin deficiency. Greene[554] reported that rachitic rabbits showed a greater morbidity and mortality from intranasal inoculation with Type I Pneumococcus than did normal controls. Differences in the susceptibility of rabbits of diverse breeds has also been noted; hence, in the selection of rabbits for comparative or quantitative tests on the virulence of strains or types of pneumococci or for measuring the potency of immune serum, consideration should be given to the breed, age, weight, and diet of the test animals.

THE GUINEA PIG

The comparatively low and variable susceptibility of guinea pigs precludes their use for many purposes in experimental studies on Pneumococcus. The susceptibility of the guinea pig to pneumococci administered by inhalation was first demonstrated by Neufeld and Ungermann[1001-2] in 1912. The inoculation, in some cases but not regularly, produced a slow pneumonic process. When the culture was injected directly into the lung, the animals developed acute infection, dying in one or two days from pleuropneumonia or bronchopneumonia.

The intrapleural injection of pneumococci into guinea pigs may cause a pleuropneumonia, as shown by Kolmer.[741] Neufeld and Ungermann[1001-2] and also Engwer[365] reported similar successful in-

fection after the injection directly into the lung of pneumococci cultivated by several animal passages.

One objection to the use of these animals is their liability to develop spontaneous pneumococcal infection, to become carriers, and thus to transmit infection to other laboratory animals. Such an outbreak was reported in 1922 by Gheorgiu,[512] but no mention was made concerning the type to which the infecting organism belonged. The presence of pneumococci as secondary invaders in an epidemic among guinea pigs and mice caused by *B. bronchisepticus* was observed by Keegan.[700] Branch[147] in 1927 reported the presence of pneumococci of Group IV in all of thirty-six guinea pigs in a laboratory epidemic. The organism failed to agglutinate with specific immune serum for the first three fixed types, but by its protein fraction appeared to be related serologically to strains of pneumococci of human origin. The infection took the form of otitis, enlargement of the spleen, lobular and even true lobar pneumonia. According to Bruckner and Galasesco as well as to Chevrel and Ranque,* spontaneous epidemics sometimes begin with septic abortion of the guinea pigs.

The presence of pneumococci in the nares of apparently normal guinea pigs and of guinea pigs affected with snuffles, and the occurrence of natural epidemics of pneumococcal infection in animals of this species, was investigated by Neufeld and Etinger-Tulczynska[984-5] in 1931. The organisms responsible for natural infections, including those found in a previous investigation of an outbreak in another colony of animals, when tested by means of the *Quellung* phenomenon, proved to belong to Type XIX. The strain was only slightly virulent for mice and guinea pigs. The authors found further that animals surviving the intranasal implantation of pneumococci of Types I and XIX, as a rule, become carriers of the respective strains.

Neufeld and Etinger-Tulczynska also noted wide variations in the susceptibility of the animals to natural infection from pneumo-

* Quoted by Neufeld and Schnitzer.

cocci and, while cold, avitaminosis, and pregnancy had previously been shown to diminish resistance of guinea pigs, the authors concluded that higher susceptibility was apparently of a more obscure nature and was associated, as earlier suggested by Uchida,[1432] with basic or temporary variations in the disposition of individual animals. A possible explanation for some of the constitutional differences in the susceptibility of guinea pigs is to be found in the communication of Nicholls and Spaeth (1922),[1005] according to whom there is a definite correlation between pigmentation and resistance to infection. White-coated, pink-eyed guinea pigs, probably pure albinos, were found to be far more susceptible to a given Type I culture than were pigmented individuals. It seems unlikely that pigmentation of itself was responsible for the resistance, although it may well have been associated with the true cause. Uchida,[1431] who earlier had isolated pneumococci from guinea pigs suffering from spontaneous infection, noted irregularities in the results following the subsequent subcutaneous, intraperitoneal, and intravenous injection of the strains into normal guinea pigs. The author assumed that the discrepancies in the outcome of the experiments were due to varying degrees of resistance possessed by the individual animals, but offered no specific explanation for the differences.

Wámoscher (1927)[1479] observed that scurvy and chronic tuberculosis in the guinea pig were diatheses favoring spontaneous pneumococcal infection. These and similar debilitating conditions may likewise lower resistance to experimental infection. Schmidt-Weyland and Költzsch* found that scorbutic guinea pigs could easily be infected when pneumococci were introduced into the body by inhalation or feeding.

THE MOUSE

The white races of the mouse family, because of low cost, ease of handling, great susceptibility, and general uniformity of reaction,

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* Quoted by Neufeld and Schnitzer.

are ordinarily chosen for the isolation of pneumococci, the pre-
liminary testing of antigenic substances, and for the determination
of type-specificity and potency of diagnostic and curative immune
serums. Albino strains are preferable to strains of pigmented or
wild varieties because of their lower resistance to infection. Pure-
line races, with their inherited uniformity of susceptibility, would
be ideal types for routine investigative purposes were it not for
their present scarcity and prohibitive cost. That susceptibility or
resistance to infection are transmissible characters and that the
breeding of races with either high or low degrees of susceptibility
can be accomplished by proper selection has been demonstrated by
Irwin and Hughes[670] in the case of the rat for bacteria of the en-
teric group.

The extreme susceptibility of the mouse to subcutaneous, intra-
peritoneal, or intravenous injection of Pneumococcus is shown by
the rapid invasion of the blood stream and the death of the ani-
mals without localization of the infection. Introduced by any of
these routes Pneumococcus may be highly infective for mice. When
the virulence of a strain of Pneumococcus has been exalted by suc-
cessive mouse passage, the cultures used for intraperitoneal injec-
tion into a mouse may be so diluted that, although the amount used
for inoculation may yield only one or even no colonies, infection
will frequently follow. In fact, Wámoscher[1478] has demonstrated by
micromechanical isolation of single cells that one pneumococcus
may suffice to infect a mouse. It is for this reason, therefore, that
the mouse is so admirably adapted for the detection of pneumo-
cocci in infective material, for determining virulence, for testing
the immunizing action of antigens, and for measuring the protec-
tive power of specific immune serum.

An observation concerning the dominance of one type of Pneu-
mococcus in causing general infection in the mouse following the
injection of an inoculum containing pneumococci of several types
was described by Etinger-Tulczynska.[368] When mixtures of equal
parts of cocci of different types were administered either by the

subcutaneous, intraperitoneal, or intravenous route or by all three routes simultaneously, the organisms of one type would gain the ascendancy and suppress the pneumococci of the other type even at the site of inoculation.

Webster and Clow[1494] found individual differences in mice to intranasal infection with Pneumococcus. Some mice were completely refractory, some became carriers, and others developed various forms of infection ranging from lobar pneumonia to septicemia. Animals which showed high resistance to a strain introduced through the nose might exhibit moderate or high susceptibility to the same strain injected intraperitoneally. Whether the grades of infectivity were due to differences in the virulence of the organisms or to degrees of susceptibility of the host was not ascertained.

Distinct differences in the reactivity of mice of diverse races to pneumococcal infection has been demonstrated by Rake.[1117-8] In general, reactivity was found to be influenced by the type of organism used for inoculation as well as by innate characters peculiar to the breed of the animal. For example, a single type of Pneumococcus produced in mice of the same breed lesions which were similar and predictable. Lesions differing quantitatively could be produced in various breeds of mice by inoculation of the same type of organism, but inoculation of cultures of the various types into mice of a single race produced lesions differing in quality. Experiments in which mice were infected intranasally and intravenously revealed that lesions in the lung and other organs varied with both the type of culture and the strain of mice used.

The constancy of the results obtained in infection experiments with this animal species is further conditioned by the age of the individuals employed. Moreover, there is greater regularity in the behavior of mice of approximately sixteen to twenty-one grams in weight to artificial infection, and this fact is especially significant when mice are employed for testing the potency of antipneumococcic serum. More recently, Goodner and Miller (1935),[540] extending Goodner's work on the rabbit, investigated the physiological vari-

ables responsible for the resistance of the individual mouse to pneu-
mococcal infection by studying the capacity of animals of a sin-
gle strain to utilize the protective properties of antipneumococcic
serum. The authors concluded that the important variables were
body-weight and the number of cells in the peritoneal cavity fol-
lowing the intraperitoneal injection of pneumococci and homolo-
gous immune serum, which in turn resolved itself into the number
of monocytes present. The dominant factor in determining sus-
ceptibility, other factors being equal, was the relation between the
number of monocytes and the number of pneumococci in the peri-
toneal cavity at the time of the injection of culture and serum.

The resistance of mice to infection by inhalation may be de-
creased by the previous administration of alcohol. Stillman and
Branch (1924, 1930, 1931)[1337, 1340-1] found that inspired pneumo-
cocci rapidly disappeared from the lung of normal mice and rarely
caused septicemia, but in alcoholized mice the organisms persisted
in the lung for a longer period and fatal septicemia was frequent,
while pulmonary localization of the infection occurred in mice pre-
viously immunized either actively with heat-killed pneumococci or
passively with homologous immune serum. The observations of
Branch and Stillman[148] were confirmed by Lange and Keschis-
chian,[785] who succeeded, but only with difficulty, in inducing pul-
monary infection in mice through inhalation. The latter authors
also found mice to be resistant to percutaneous and peroral inocu-
lation with pneumococci.

The frequency with which colonies of white mice are infected
with the so-called mouse typhoid due to *Bacillus typhi murium* is a
factor to be considered when selecting mice for experimental pur-
poses. The disease, so often remaining latent, may not be evident,
but it may nevertheless greatly alter the reaction of the animals to
experimental inoculation.

THE RAT

The rat is a near zoological relation of the mouse and is highly

receptive to pneumococcal infection, but it is seldom used in researches on Pneumococcus. Lamar* and also Neufeld and Haendel* proved the great susceptibility of the rat to pneumococcal infection. The subcutaneous injection of 10^{-5} cubic centimeters of a virulent strain caused septicemia which resulted two days later in death of the animal.

McDowell[878] studied the effect of high air temperatures, combined with different degrees of humidity, on resistance of rats to intraperitoneal inoculation of pneumococci. It appeared that after an exposure for two weeks to a temperature of 83°F., with the humidity varying from 44 to 72 per cent, rats exhibited greater resistance than did rats kept at medium temperatures. High or low humidity with temperatures between 65° and 72°F. were unfavorable to the survival of the test animals. However, when rats accustomed to moderate temperatures (67° to 71°F.) were inoculated intraperitoneally with Pneumococcus and then exposed to a higher temperature (83°F.) there was a lowering of resistance.

Rats, as well as mice, proved to be susceptible to pneumococcal infection when the organisms in the form of pulverized, dried, infected blood or spleens were insufflated into the trachea by Kramár and Gyüre.[757] McDaniels[876-7] employed rats in an investigation of the immunizing action of orally administered pneumococcal vaccines and found the species to be suitable for the purpose.

THE MONKEY

Monkeys in captivity are susceptible to spontaneous pneumococcal disease. Blake and Cecil (1920)[128] reported that pneumonia might occur in epidemic form, due to spread of infection from animal to animal, when conditions favoring close contact existed. The disease in monkeys, according to the authors, was identical in its clinical features, complications, and pathology, with lobar pneumonia experimentally produced in monkeys by the intratracheal injection of Pneumococcus and with lobar pneumonia in man. Two

* Quoted by Neufeld and Schnitzer.

strains of the organism isolated from the sick monkeys were pneumococci of Group IV. At that time, the authors[126-7] demonstrated that lobar pneumonia could be consistently produced in members of this animal species (*Macacus syrichtus* and *Cebus capucinus*) by the intratracheal injection of minute amounts of culture of a virulent Type I strain. When large quantities of culture were introduced into the nose or throat, no lobar pneumonia developed, but the animals became carriers of the inoculated organism and remained so for a period of at least a month.

The susceptibility of the monkey (*Macacus rhesus* and *Ceropithecus callitrichus*) to intracranial or intraspinal inoculation with Pneumococcus was demonstrated in 1912 by Lamar,[775] who described the meningitis following the injections. The experimental disease resembled pneumococcal meningitis in man but ran a more rapid course and was invariably fatal.

In studying pneumococcal infection and immunity in monkeys, Cecil and Steffen[211] found that *Macacus rhesus* was most resistant to Type I Pneumococcus and rarely developed true lobar pneumonia; *Cebus capucinus* occupied an intermediate position, occasionally showing typical lobar infection but more often interstitial or patchy lesions; while the Philippine monkey, *Macacus syrichtus*, was the most susceptible and was preferable for inoculation experiments because animals of that species develop true lobar pneumonia.

The existence of the carrier state in normal stock monkeys was also shown by Schöbl and Sellards,[1246] who recovered an avirulent strain of Pneumococcus from the Philippine monkey, *Pithecus philippinensis*. The authors also succeeded in inducing pneumonia in the animals by intratracheal inoculation with a small dose of a broth culture of Type I Pneumococcus that was highly virulent for mice.

Blake and Cecil,[126] using strains of Type I, II, III, and Group IV pneumococci, by intravenous injection failed to produce pneumonia in monkeys of the *Macacus syrichtus* species but reported

that a fatal septicemia followed inoculation by that route. No greater success followed the attempts of Schöbl and Sellards, although the latter authors likewise observed fatal systemic infection after intravenous injection of a Type I culture. Cecil and Steffen[214-5] were able by injecting cultures of Types I, II, and III, and of one Group IV strain into the trachea to incite pneumonia in *Macacus rhesus, Macacus syrichtus,* and *Cebus capucinus.*

Among other references to the experimental production of lobar pneumonia in monkeys by the intratracheal implantation of virulent pneumococci may be mentioned the report of Francis and Terrell,[476] who were able to induce pneumonic disease in *Cynomolgos* monkeys with a Type III strain. The authors reported that the type of infection following small doses given in winter was similar to the type resulting from large doses administered in warm months. The authors also noted marked individual variations in the monkeys used. According to Stuppy, Falk, and Jacobson,[1353] *Macacus rhesus* and *Cebus capucinus* were highly resistant to intratracheal inoculation of virulent Type I Pneumococcus. None of the thirteen animals injected developed lobar pneumonia. Three monkeys died from pneumococcal infection but the lungs appeared to be normal, except for an increase in the number of polymorphonuclear leucocytes in the interstitial tissue, blood vessels, and bronchi.

THE CAT

Feline animals are unsuitable for studies on pneumococcal infection. Robertson, Woo, Cheer, and King[1152] are apparently the only authors to have reported experiments on cats in which it was possible by intrapleural injection of cultures of Type I and II pneumococci to produce lobar pneumonia. However, only one animal of those inoculated developed the disease.

THE DOG

The first reference to the use of dogs in research on Pneumococc-

cus is probably that of Friedländer (1883),[487] who injected aqueous suspensions of gelatin cultures of cocci isolated from pneumonia patients into the lungs of four dogs, only one of which succumbed. The animal at necropsy showed red and gray hepatization of the lung, and from the areas Friedländer succeeded in recovering typical encapsulated diplococci. In the same year, Talamon[1378] failed to infect dogs with a mixed culture containing lanceolate cocci grown from the exudate of a pneumonic lung. The refractoriness of dogs was also demonstrated by Monti,[905-7] who reported that no reaction attended subcutaneous injection. However, he succeeded in producing meningitis in the dog after subdural inoculation with pneumococci.

Salvioli (1884)[1215] was more successful in infecting dogs with encapsulated cocci obtained from the pleural and pericardial exudates of pneumonia patients, but the animals so treated failed to develop the typical lesions of pneumonia. In 1912, Lamar and Meltzer,[772] by means of a catheter introduced through the larynx and bronchus, implanted pneumococci in the lungs of dogs. Of forty-eight test animals, forty-two developed lobar pneumonia with a fatality rate of 16 per cent. Wadsworth[1457] employed the method to study phagocytosis in similarly infected animals. In the next year, Wollstein and Meltzer[1539-40] in two communications described the results following the insufflation of avirulent and of heat-killed pneumococci into the lungs of this domestic animal. The injection caused pulmonic congestion with exudate but, in general, the framework of the lung was unaffected and the process was non-fatal.

Similar results were reported by Newburg, Means and Porter (1916),[1004] by Kline (1917),[726] Wadsworth (1918),[1459] Leake, Vickers and Brown (1924),[793] Christie, Ehrich and Binger (1928),[233] and by Coryllos (1929),[279] all of whom employed the same technique of using the bronchoscope for the injection of Type I culture as used by Henderson, Haggard, Coryllos, and Birnbaum (1930)[636] with a culture of Type II Pneumococcus.

With strains of both Types I and II introduced into a terminal bronchus by means of a catheter, Terrell, Robertson, and Coggeshall (1933)[1387] made fluoroscopic studies of the lobar pneumonic process which ensued in every instance when appropriate doses of culture were administered. In the experiments of the last-named authors the course of the pneumonia was short, averaging four or five days, while recovery, which took place in the majority of the animals so infected, was abrupt and simulated the crisis occurring in man. Similar experiments with pneumococci of Types I and III were described in 1934 by Lieberman and Leopold.[813] The article by Terrell, Robertson, and Coggeshall[1387] contains excellent descriptions of the pathological processes observed following intrabronchial injection of pneumococci in dogs.

Bull (1916)[173] injected dogs intravenously with pneumococci. Invasion of the blood stream appeared twenty-four to forty-eight hours after the injection. The septicemia reached a climax in four to five days, then abruptly declined, the blood becoming sterile in one to three days after the peak of the septicemia was reached. In some of the animals so injected meningitis occurred.

The results of a study of meningeal inoculation in dogs have been described by Stewart.[1323] The injection of Type I and Type II organisms into the ventricle, the cistern, and the lumbar region was followed by purulent meningitis with accompanying bacteriemia when the cultures used were of high virulence. Not all the animals infected succumbed, but those that died showed pathological changes not entirely comparable to those found in pneumococcal meningitis in man.

THE HORSE

The susceptibility of horses to infection with Pneumococcus, mentioned by Neufeld and Schnitzer, is a fact all too familiar to those engaged in the manufacture of therapeutic antipneumococcic serum. The possession of a high degree of specific, active immunity to pneumococci of a given serological type may fail to

protect the horse against the subcutaneous or intravenous injection of living, virulent strains of the same type. Abscesses, pneumococcemia, endocarditis, and pneumonitis, sometimes culminating in fatal pneumothorax, may follow immunizing injections of living cultures, even when the serum of the animal under active immunization contains specific agglutinins and protective antibodies in sufficient quantity to qualify it for therapeutic use. This paradoxical phenomenon will be discussed in a later chapter.

BIRDS

Hens and doves have been found to be refractory to infection with Pneumococcus by Fraenkel,* Gamaléia,[498] and by Kyes,[766] among others. Kindborg (1905)[713] apparently was the only worker who claimed to have succeeded in demonstrating pathogenicity of Pneumococcus for pigeons. Kyes studied the cellular reaction in tissues of pigeons injected intraperitoneally with virulent pneumococci, and reported that the invading organisms were rapidly withdrawn from the blood stream and localized in the liver and spleen. Because the ultimate localization of the cocci in both of these organs was within a type of fixed phagocyte—the hemophage—common to both organs, Kyes concluded that the phagocytic destruction of pneumococci by hemophages is so extensive and so rapid as actually to constitute an important, if not indeed the determining, factor in the resistance of birds to pneumococcal infection. The natural resistance of hens and doves may, however, be lowered to the point of susceptibility through vitamin deficiency or by the administration of poisons, as reported by Strouse, by Guerrini, and by Corda.*

Virulence of the Organism

In any discussion of the pathogenicity of an organism, it should be borne in mind that virulence is a purely relative term. The factors which enter into the determination of the invasiveness of a

* Quoted by Neufeld and Schnitzer.

given strain of Pneumococcus are the serological type; the vital condition of the coccus at the time of trial as shown by the possession of a capsule; the mass of culture injected; the site chosen for inoculation; the species, and even the variety and the individual idiosyncrasies of the animals selected. There also enters the question whether the malignancy of a culture is due to a uniform virulence of all the cocci in a culture or to the presence of a few organisms of especially high virulence accompanied by other organisms possessing less or no invasive power.

Pneumococci of the various serological types as they exist in the lesions, exudates, or secretions of infected animals, or when freshly isolated from these sources, are pathogenic for other animals of the same species and, depending upon the serological type, may be virulent for animals of a different species. When a strain is passed serially through the bodies of animals of a given species it may acquire an elevated infectivity for other members of that species. Virulence may reach a permanent zenith or may decline but, virulent though an organism may be for the species used, it by no means follows that it is correspondingly pathogenic for animals of a different species.

During propagation outside the body the conditions of cultivation effect profound changes in the integrity of Pneumococcus, which, in turn, affect its pathogenic powers. Conditions that serve to maintain the organism in the highest state of metabolic activity with maximal production of capsular substance favor increase and maintenance of virulence. Conditions that, on the contrary, induce dissociation and degradation of the organism, by being inimical to capsule formation, lessen or destroy virulence.

FRESHLY ISOLATED STRAINS

In considering some of the earlier reports dealing with the pathogenicity of strains isolated from cases of lobar pneumonia and other pneumococcal infections, it should be remembered that many of the studies were conducted at a time before pneumococci

were divided into serological types. Eyre and Washbourn[376] in 1899 gave descriptions of four strains of pneumococci, of which three, isolated from infections in man, were considered by the authors to be parasites, and one strain, from the normal human mouth, was looked upon as a saprophyte. The first three strains displayed great capabilities both for acquiring and for retaining a high degree of virulence, whereas the fourth culture possessed a low capacity in both respects.

The severity or lack of severity of an infection may depend on a preceding or accompanying infection with another bacterial species. For example, Sinigar (1903)[1292] described the ascending virulence of a respiratory infection among the staff and patients in the Leavesden Asylum. Beginning as a brief, indefinite illness, the disease gradually increased in severity, occasionally showing bronchial symptoms, and then developed into lobar pneumonia with a high fatality-rate. In all the cases, it was alleged that pneumococci were present in large numbers but, since there is no reference in the text to cultivation or virulence tests, it is impossible to say whether the same strain of Pneumococcus—if the organism was a pneumococcus—gradually gaining virulence, was responsible for the epidemic, or whether pneumococci of relatively low virulence were succeeded by a type having greater infectivity.

Kindborg (1905),[713] in a study of a large number of strains obtained from normal and pneumonic sputum, empyema pus, and other sources due to pneumococcal infection, observed wide limits in the pathogenicity of the different cultures. The organisms from cases of pneumonia were said usually to be the most virulent, while strains isolated from local inflammatory processes were generally avirulent. Whittle[1519] determined the virulence for mice of sixty strains of pneumococci collected at random from infections in man, and concluded from his tests that pneumococci by virtue of their pathogenic powers could be divided into at least two groups; those of high virulence were responsible for such well-recognized clinical

entities as lobar pneumonia and bronchopneumonia, and those of low virulence were associated with minor illnesses or with disease occurring in persons already debilitated. Whittle denied the existence of any relation between serological type and pathogenicity.

Gundel and Wasu (1931)[578] reported that the virulence of a given type of Pneumococcus reached a maximum at the height of the disease process and that in the complications of lobar pneumonia in man, such as meningitis and otitis media, only strains of the highest virulence were found, the organisms usually belonging to the fixed serological types. Further discussion of the infectiousness for man of pneumococci of the various serological types will be found in the following chapter.

NUMBERS OF COCCI REQUIRED TO INFECT

Among reports on the quantitative determination of the infective ability of pneumococcal strains there may again be mentioned the observations of Wámoscher (1926).[1478] Using a strain of Type III Pneumococcus, counting the number of cocci in the inoculum by means of the Peterfi micromanipulator, and injecting the organisms subcutaneously into white mice, the author found that approximately one-quarter of the number of mice so treated succumbed to pneumococcal infection in two to four days after receiving an inoculation of one pneumococcus. The percentage of fatal infections rose with an increase in the number of organisms injected. Using only freshly isolated strains of pneumococci grown in broth from the culture obtained from a single mouse passage after isolation from a human source, and injecting the culture intraperitoneally into white mice, Gundel and Wasu noted marked differences in the virulence of strains within a type, although, as a rule, representatives of Types III, II, I, and IV possessed degrees of virulence in the order named, the average minimal lethal dilution for all Type III strains being 1 to 100,000,000, for Type II cultures 1 to 10,000,000, for Type I cultures 1 to 1,000,000, while

for pneumococci of Type IV the killing dose varied from 1 to 100 to 1 to 1,000. One of the most virulent strains tested, a Type IV culture, was fatal in a dose of 10^{-8} cubic centimeters.

Petrie and Morgan[1085] in the same year reported the results obtained in an investigation of the factors influencing the lethal power for mice of a virulent culture of Type I Pneumococcus. Stocks of mice from different sources appeared to show some variation in susceptibility, and a small proportion—from 5 to 10 per cent—exhibited an innate resistance to small doses of pneumococci. The weight factor of the test animals failed to affect the mortality, although it modified the survival time. However, the authors decided that the density and virulence of the culture determined its lethal power, and that this power could be calculated when a reasonable number of mice were used. The criteria, by means of which the minimal fatal dose of any culture of Type I Pneumococcus could be specified, were the percentage fatality, the mean death-time, and the distribution of deaths at appropriate time intervals in a group of mice receiving a definite dose of the test culture.

AVENUE OF INOCULATION

In general, the virulence of a given organism varies with the tissue into which it is introduced. Direct inoculation into the blood stream is the most rapidly and most surely lethal route, followed in order of effectiveness by injection into the anterior chamber of the eye, into the peritoneum, under the skin, into the skin, into the lung either by direct puncture or through the bronchi, and lastly by way of the mouth.

DETERMINATION OF VIRULENCE

While the intraperitoneal injection of white mice is the preferred method for testing the virulence of pneumococcal cultures, other methods have been described. The rabbit, because of cost and individual and special idiosyncrasies, is less valuable. The relation

between virulence and electrophoretic potential of different strains and serological types of Pneumococcus has been studied by Falk and his associates, Gussin and Jacobson.[379] According to the authors the highest potentials were uniformly found for Type III organisms, while the most probable sequence of decreasing potentials was from Type III to I to II and then to Group IV strains. The order corresponded to the decreasing sequence of virulence of the cultures for white mice. Washing the cultures increased electrophoretic velocities, but the order remained the same. From the results obtained with stock cultures, the authors anticipated that strains isolated from fatal cases of pneumonia might show higher potentials than would strains of the same type isolated from nonfatal cases. The experimental data confirmed the assumption for Types II and III, and Group IV, but contradicted it for Type I strains. Falk, Gussin, and Jacobson[379] concluded that electrophoretic potential was related in some fundamental manner to virulence as well as to phagocytability, agglutinability, capsule formation, and other characters of microorganisms. In a later communication, Jacobson and Falk,[675] after a study of this electrical phenomenon in pneumococcal variants, reported that in all cases studied alterations in virulence were accompanied by parallel alterations in electrophoretic potential and by reciprocal alterations in agglutinability.

The results obtained by Thompson (1931)[1396] in some respects contradict those reported by Falk and his colleagues. While the electrophoretic migration of representatives of the first three pneumococcal types was observed to decrease in the order III, I, II, as previously noted by Falk, Gussin, and Jacobson, exaltation or degradation of virulence respectively by mouse passage and by growth in increasing concentrations of ox bile were unaccompanied by any constant differences in the original velocity of migration.

Welikanow and Michailowa (1930)[1513] claimed that variation in the ability of pneumococci to ferment glucose is an indicator

of the virulence of the strain, but the evidence presented is too meager to be convincing.

In Chapter III mention was made of substances or preparations which possessed the ability to increase the invasive power of pneumococci. The virulin of Pittman and Falk,[1092] the extracts of Pittman and Southwick,[1093] the leucocidin of Oram,[1032] and the toxic autolysate of Parker and Pappenheimer[1063] are examples of substances which to a greater or less degree possess this property. A similar action was observed by Sia,[1266] who found that the addition of a very small amount of soluble specific substance or of young broth cultures of pneumococci to avirulent cultures of homologous type when mixed with rabbit or cat serum-leucocyte mixtures favored the growth of the organism. The authors interpreted the result as indicating that soluble specific substance had the power of rendering virulent an avirulent Pneumococcus of the same serological type. Sia and Zia[1275] reported that the injection of Type II soluble specific substance into rabbits depressed the resistance to such an extent that the animals succumbed to the intravenous injection of an otherwise sublethal dose of pneumococci of the same serological type. The authors left undecided the question whether the result was to be ascribed to a heightened susceptibility of the animal or to an enhanced virulence of the organism.

A somewhat similar effect was obtained by Nungester, Wolf, and Jourdonais,[1020] who added gastric mucin to twenty-four-hour broth cultures of Type II pneumococci and injected the mixture intraperitoneally into mice. Control mice received similar injections of the culture suspended in saline instead of mucin solution. At only one dose level was any marked difference noted in the percentage of survivals among the test and the control animals. The effect was not apparent when the injections were made intravenously or subcutaneously. Any action of mucin, in spite of its

correspondence in chemical structure to soluble specific substance of Pneumococcus, must have been non-specific, and this surmise is strengthened by the fact that a slight adjuvant action in favoring infectivity was also noted when streptococci instead of pneumococci were the organisms so treated and inoculated.

The influence of pneumococcal autolysates on the invasiveness of pneumococci injected intradermally into rabbits was studied by Goodner. Whereas autolysates favored the pathogenicity of the culture used, they failed to alter its virulence. The evidence, taken as a whole, favors the hypothesis that the action of culture filtrates, extracts, autolysates, or specific capsular polysaccharides in increasing the infective power of pneumococci is to interfere with the natural defensive processes of the body and thereby lower the host's resistance rather than to add to the virulence of the cell itself.

CULTURAL CONDITIONS AND VIRULENCE

Influences that stimulate the anabolic processes of the pneumococcal cell make for virulence. Enrichment of the cultural medium with blood, normal serum, or other growth accessories, the proper concentration of hydrogen ions, and optimal temperature of incubation enable the organism to elaborate capsular substance, which, as will be shown in other portions of the present volume, may determine the invasive power of a given strain. Frequent transplantation in favorable media of pneumococcal cultures taken at the period of maximal growth not only maintains but increases virulence (Wadsworth and Kirkbride[1471]). Repeated transfer by means of the automatic device of Felton and Dougherty[423] of young cultures of a pure-line strain of Type I Pneumococcus into a fresh supply of sterile skimmed milk caused an avirulent strain to acquire a virulence ten million times greater than that of the original culture. Within a wide range, the hydrogen ion concentration of the milk appeared to have only a slight effect on virulence when transplants were made at two-hour intervals. However,

when the reaction of the medium was adjusted to pH 8 or 9, viru-
lence decreased; the more alkaline the reaction the more rapid the
decrease.

When cultivated in broth by the same method,[425] the Type I cul-
ture lost virulence as the reaction of the medium by adjustment
became more acid. Virulence fluctuated with the frequency of trans-
fer of the organism to fresh broth. In the case of transplants made
every hour, virulence immediately decreased. When the interval
was two, four, or eight hours, there was first a rise and then a fall
of virulence, the rise being greatest in the case of eight-hour and
least with the two-hour transfers. With the hydrogen ion concen-
tration at set points the amount of meat infusion influenced the
virulence of the culture, the unfavorable action varying in inverse
proportion to the concentration of meat in the substrate. The ad-
dition of glucose neutralized the action of the meat. Peptone in
the broth also affected the virulence of the strain studied, Felton
and Dougherty reporting that the nutrient in a concentration of
2 per cent maintained and even increased virulence of the strains
studied.

In a later communication (1932), Felton[411-2] described other
nutritional factors that affect the virulence of pneumococci. Media
made from calf lung or heart or from the skeletal muscles of the
horse maintained for a long period of time the virulence of the cul-
tures used; conversely, media prepared from calf spleen led to a
decrease in pathogenicity. Normal horse serum, or specific immune
serum freed from protective antibody, preserved virulence. Media
made from rabbit muscle were less suitable for the purpose than
media prepared in the same way from the meat of guinea pigs.
When grown in the automatic transfer device in a medium which
aerobically maintained the virulence of a Type I culture, the addi-
tion of pure oxygen or pure carbon dioxide lowered virulence, but
no change in infectivity was noted when the organisms were culti-
vated in the presence of nitrogen. Gradual increase in the tem-
perature of incubation from 36.5° to 42° over a period of ten

days, in a sample of medium otherwise suitable for maintenance of virulence, resulted in a decrease in infective power of the pneumococci studied.

According to Gaskell,[499] a medium made from beaten eggs was satisfactory for preserving the virulence of pneumococcal cultures, but the interpolation of fortnightly passages through the mouse was necessary in order to restore waning infectivity. For additional methods for assuring or preserving the virulence of cultures of Pneumococcus or of fluids or tissues containing the organism, the reader is referred to Chapter II.

STRAIN VARIATIONS IN VIRULENCE

The ability of a given strain of Pneumococcus to retain its virulence over a long period and then suddenly to lose its invasive power is too well known to require extended comment. Browning and Gulbransen (1923)[161] described a culture of Type I Pneumococcus, passed more than ninety times through mice during a period of six years, which, when preserved in the dried spleen of an infected mouse, exhibited marked variations in its ability to infect mice. The conclusions reached by Gaskell (1928)[501] in a study of the pathogenicity of single strains of pneumococci of Types I, II, and III and Group IV may be cited. The virulence of Type II strains was less than that of Type I organisms for both mice and rabbits; the pathogenicity of members of Type III was lower than that of Type I for rabbits, mice, and also man; whereas the virulence of the Group IV cultures obtained from severe infections in man was, if anything, higher than that of Type I pneumococci.

Other individual strain differences with respect to the ability of pneumococci to infect experimental animals have been reported by Webster and Clow (1933).[1494] The degree of virulence of a strain when inoculated into the nose of the mouse failed to parallel intraperitoneal virulence in 50 per cent of the strains studied—high intranasal invasiveness being accompanied by either high or moderate intraperitoneal virulence, and low intranasal by high, mod-

erate, or low intraperitoneal virulence. Type III strains were of relatively high intranasal and intraperitoneal infectivity; Type II organisms were low in intranasal but high or moderate in intraperitoneal virulence; Type I strains were all low in intranasal but either high or moderate in intraperitoneal virulence; while the majority of strains of other types were low both in intranasal and intraperitoneal pathogenicity. Intranasal virulence of pneumococci was not enhanced by animal passage, but nasal passage reduced the intranasal virulence to zero without altering intraperitoneal virulence, colony form, or agglutinative specificity of the strains. Passage by the intraperitoneal method maintained the characteristic level of intranasal virulence for a period, increased intraperitoneal virulence in some instances, but did not affect colony form or agglutinative properties.

VIRULENCE IN RESPECT TO ANIMAL SPECIES

In addition to individual differences in pneumococci, variations in the susceptibility of various animals are factors to be reckoned with in the evaluation of the virulence of a given type or strain. An organism may possess superior virulence for animals of one species and yet fail to infect those of another species, although the animals may readily be infected with representatives of a different variety or type. Eyre and Washbourn,[374] in 1898, reported that, after increasing the virulence of a culture for guinea pigs by repeated passage through a series of these animals, there was no change in the virulence of the organism for mice. Fourteen years later, Neufeld and Ungermann[1001-2] observed that while repeated passage of a strain of Pneumococcus through the guinea pig elevated the virulence of the organism for animals of the same species, similar serial propagation in mice failed to increase the infectiousness of the strain for the guinea pig.

In 1912, Truche and Cotoni[1423] reported that strains of pneumococci isolated from human sources were rarely virulent for rabbits or guinea pigs. Strains virulent for rabbits were always infective

for mice, while pneumococci that were invasive for guinea pigs possessed high virulence for both rabbits and mice. In a second paper, Truche and Cotoni[1424] described the effect of passing four strains of Pneumococcus through mice, rabbits, and guinea pigs. When strains virulent for mice were serially propagated through animals of that species the organisms maintained their high virulence but acquired no greater virulence over the original culture for rabbits or guinea pigs. The same condition held true when pneumococci were passed through rabbits, but there was no alteration in the infectiousness of the strains for guinea pigs or mice. Cultures subjected to passage through guinea pigs showed no increase in pathogenicity for animals of that species or for mice or rabbits.

Twenty strains of Type III Pneumococcus studied by Lévy-Bruhl (1927)[804] were highly virulent for guinea pigs, moderately so for mice, and very feebly or not at all virulent for rabbits. The failure of many strains of Type III Pneumococcus to infect rabbits was also reported in the same year by Tillett.[1403] Of eleven strains isolated from human sources, ten possessed low virulence for rabbits despite the fact that all the strains possessed large capsules and a high degree of virulence for mice. The odd strain was rendered highly infectious for rabbits, and since it possessed no other biological property demonstrably different from the other ten strains, Tillett concluded that its individual virulence must reside in some additional property.

The lack of virulence for rabbits of the majority of Type III pneumococci has perplexed many bacteriologists, and no explanation has been forthcoming for the disparity in virulence of Type III strains until the recent communication of Enders and Shaffer.[360] Evidence was obtained that a correlation exists between the inability of a strain to grow at 41° and virulence, since only among the thermoresistant strains were found those possessing the property of rabbit virulence. The attribute is constantly but not exclusively associated with all Type III pneumococci, and was con-

sidered by the authors as a prerequisite, but not the sole factor, in determining the virulence of an organism for rabbits. Continuing the investigation, Shaffer with Enders and Wu[1259] studied two smooth strains of the same organism, one virulent and the other relatively avirulent for rabbits. Although no antigenic differences between the two organisms were revealed by cross-absorption of agglutinins from homologous antiserum, the latter strain lost its capsule in dextrose serum-broth cultures about eight hours earlier than was the case with the rabbit-virulent organism. With the loss of capsule there was marked shrinkage in volume, alteration in the zone of acid agglutination, susceptibility to agglutination in antirough pneumococcic serum and to phagocytosis.

The results which followed the intravenous injection into rabbits of the two strains varied with the state of the capsule. A culture of either strain became susceptible to the blood-clearing mechanisms contemporaneously with the onset of capsular degeneration and the beginning of other concomitant changes at the surface of the organism which occurred much earlier with the less virulent strain. Phagocytosis by the leucocytes of the normal animal either *in vitro* or *in vivo* was observed only at such a time as the capsule had become impaired. The authors (Enders, Shaffer, and Wu)[361] concluded that virulence for rabbits of the two strains of Type III Pneumococcus does not imply that this animal possesses a defensive mechanism which is absent in other species, since it was possible to demonstrate similar differences in virulence when the organisms were injected intravenously into mice. Therefore, "the factors determining the degree of virulence of these strains are to be sought in the organisms themselves, rather than in the kind of host."

In a fourth publication of the series, Enders, Wu, and Shaffer[363] discovered that the addition of the C Fraction of Tillett and Francis[1409] to serum-leucocyte mixtures decreased the phagocytosis of both rabbit-virulent and avirulent strains of Type III by the cells and serum of both man and the normal rabbit. Furthermore, the

addition of antirough pneumococcic serum to normal rabbit serum and cells resulted in increased phagocytosis of the strains that had been partially inhibited by the C Fraction; and antirough pneumococcic rabbit serum protected mice against one hundred or more minimal lethal doses of the rabbit-virulent strain, provided the organisms were injected by the intravenous route. When antirough serum was absorbed with the C Fraction, the mouse protective property was removed. In the summary and general conclusions, the authors stated:

Since there is no evidence for the occurrence of type specific antibody in the normal rabbit and since, as we have shown, the Pneumococcus Type III whether avirulent or virulent is not removed from the blood stream or destroyed when the capsule is intact, the following factors which have been revealed in the course of our work appear to represent certain essential components, if not the complete mechanism, upon which the natural immunity of the rabbit against this organism depends. (a) The elevation of the body temperature after intravenous infection to 41°C. or thereabouts and its maintenance for varying periods. (b) The ability of the phagocytic cells, both fixed and mobile, to attack any cocci which have become vulnerable through the deterioration of capsular integrity. (c) The adjuvant effect of an antibody, reacting with the somatic C carbohydrate, which enhances the phagocytosis of such organisms as no longer possess a completely intact envelope.

Conversely, the varying degrees of virulence for rabbits observed among Pneumococcus Type III strains are based upon: (a) differences in the ability of the organisms to multiply at the elevated temperatures encountered in the infected host. Strains markedly susceptible to the harmful influence of this factor fail to induce a generalized fatal infection. Not all "thermo-resistant" strains are highly virulent, however, and these may contrast sharply with regard to (b) size of the capsule and the ease with which it is impaired or completely lost. The capsules must be maintained intact for a sufficient time until multiplication of the organisms can proceed to such a degree that death of the host results. Avirulent strains even when capable of growth at 41°C. appear to be unable to satisfy this requirement.

The differences in virulence of various strains apparently conditioned by these factors are not limited solely to the case of the rabbit, since for at least two strains similar differences in virulence have been

shown to exist when the intravenous route of infection is employed in mice.

The results of the studies by Enders and his colleagues were contemporaneously confirmed by the data obtained by Rich and Mc-Kee[1138] in an investigation on native immunity of the rabbit to Type III Pneumococcus. Strains of encapsulated Type III pneumococci virulent for mice, when injected intradermally into rabbits proliferate progressively in the tissues for some hours causing, with the dose used, a local lesion of considerable severity, often accompanied by bacteriemia. The proliferation of the cocci is then checked, the organisms are destroyed, and the animal completely recovers. During the first twenty-four hours of the infective process the rabbit acquires a greatly enhanced resistance to further infection and the increased resistance is due to the fever developing in the animal as a result of the infection. The encapsulated strains to which the rabbit is resistant are not phagocyted promptly either *in vitro* or *in vivo*, but after some hours' sojourn in the body of the rabbit, the organisms gradually lose their capsules and are avidly phagocyted. As in the body of the rabbit, the majority of the strains studied exhibited marked sensitivity to temperatures of 104° to 106° *in vitro*.

The work of Enders and his co-workers and of Rich and McKee, therefore, offers a striking example of one type of mechanism operating in a host-parasite relationship and again emphasizes the dependence of virulence upon a special physiological instead of a strictly immunological response on the part of a given animal species.

ARTIFICIAL EXALTATION OF VIRULENCE

There are several methods for raising the virulence of pneumococci. One is the frequent transplantation of the culture into a favorable medium, as advocated by Wadsworth and Kirkbride,[1471] and by Felton and Dougherty;[411-2, 423-4] another is serial passage through suitable animals, or by inoculation of animals with an or-

ganism of low virulence along with a killed culture of high viru-
lence; while another method is to grow the organism in antirough
pneumococcic serum of the homologous type. Propagation in the
body of susceptible animals has long been practiced for the pur-
pose of enhancing the virulence of pneumococci. Eyre and Wash-
bourn (1898)[374] employed the guinea pig, Truche, Cramer, and
Cotoni (1911)[1426] the mouse, while Neufeld and Ungermann
(1912)[1001-2] increased the pathogenicity of Pneumococcus, when
administered to guinea pigs by inhalation, by passage through
animals of the same species.

The procedure of serial animal passage is now too well known
to require extended comment. The intraperitoneal inoculation of
white mice with infectious material containing pneumococci or with
pure cultures, with subsequent recovery and cultivation of the or-
ganism from the heart's blood of the test animal is the method com-
monly used for raising and maintaining the virulence of pneumo-
cocci for experimental purposes or for the preparation of vaccines
for the immunization of horses in the production of therapeutic
serum as well as for testing the potency of such serums. The stimu-
lating action of the dead bodies of virulent strains of pneumococci
when injected simultaneously with living cultures of avirulent or-
ganisms of the corresponding serological type, resulting in the ac-
quisition of higher virulence, has been discussed in the chapter on
dissociation. A similar effect is produced by growth of organisms
of low virulence in media containing immune serum specific for de-
graded variants of the homologous type.

DEGRADATION OF VIRULENCE

Pneumococci as they encounter unfavorable conditions when cul-
tivated outside the animal body rapidly lose virulence. Insufficient
nourishment, excess of alkali or acid, unsuitable incubation or
storage temperatures, and infrequency of transplantation even in
a favorable medium, lower the vitality of the cell, inhibit capsule
formation, and consequently attenuate or destroy the pathogenic

power of the organism. Furthermore, there are other and specific factors which may expedite the process.

Stryker[1348] in 1916 reported that virulent pneumococci when grown in homologous immune serum fail to form capsules, show a decrease in virulence, and become more susceptible to phagocytosis. By passing the altered forms through mice, reversion to the original type takes place. Normal serum has no such effect. The experiments of Stryker, therefore, constituted the first demonstration of the ability of the specific antibodies of immune serum to inhibit the elaboration of capsular substance by Pneumococcus and consequently to render the organism vulnerable to the natural defenses of the animal body. Further developments in the investigation of the action of immune serum as well as of cultural conditions on the virulence of pneumococci have already been described in such detail, that, in order to avoid needless repetition, the reader is referred to the chapter on dissociation.

Summary

To summarize the data discussed in the present chapter, it may be repeated that in the mouse and the rabbit man has at his disposal animals admirably adapted for the several purposes of the bacteriological and immunological study of Pneumococcus. The use of in-bred strains of mice, and presumably of rabbits, reduces the frequency of irregular results in experimental work, especially in quantitative estimations of specific antibodies in immune serum. The monkey, being more closely related in the biological scale to man, presents opportunity for studying the invasiveness of pneumococci and their pathological effects in the animal economy. The horse, although obviously unsuited to the study of pathogenicity, by virtue of its size and disposition, serves as the most convenient animal for the production of antipneumococcic serum. Susceptibil-

ity or resistance depends on peculiarities due to the species, on genetic factors, age, weight, environmental conditions, and the physical state of the test animal.

In addition to the variables in the host, the invasiveness of a given strain of Pneumococcus is conditioned by its serological type, the vital condition of the culture, its mass or density, the route of inoculation, and by intrinsic factors possessed by various strains of the organism. Virulence of a pneumococcus for a given animal species may be raised by serial passage through animals of the same species, and the enhanced pathogenicity can be maintained by continued animal passage or by the application of suitable *in vitro* methods of preservation. Contrariwise, the pathogenicity of a pneumococcal strain may be decreased by subjecting the organisms to unfavorable cultural conditions or by propagating the organisms in media containing increasing amounts of homologous immune serum. Pathogenicity or virulence, therefore, is only a relative term and must be interpreted in the light of the biological characters of the pneumococcal strain and of the functional variables in the animal host. As yet no *in vitro* test has been devised which equals the use of animals, particularly the mouse and the rabbit, for studying the invasive power or virulence of Pneumococcus.

CHAPTER VII

PATHOGENICITY OF PNEUMOCOCCUS: MAN

The infectiousness of pneumococci of the different serological types for human beings; incidence and lethal powers of the cocci in disease; their distribution in the body and the lesions produced; and the various phenomena of the carrier state.

Pneumococci, if successful in passing the first defensive barriers of the human system, may become localized in a tissue or organ, mainly the lungs, whence they may invade the blood stream and under favoring conditions incite metastatic foci in other parts of the body; or, after gaining entrance, they may proceed to sites other than pulmonary tissue and there set up primary infection. The nature and severity of the infection depend on the serological type, virulence, and mass of invading cocci, the passage by which they are admitted, and on the many and varied factors which constitute natural resistance or which are involved in the creation of specific immunity in response to the presence of the invading organisms.

The epidemiology and the clinical and pathological aspects of pneumococcal infection may be left to writers competent to discuss these subjects.* Therefore, assuming that the reader of this review is more intimately interested in the vital activities of Pneumococcus as a microorganism than in the manifold morbid manifestations of the human body's response to its invasion, the present discussion is directed more particularly to the ability of pneumococci of the different serological types to incite disease in man.

Pneumococci frequently lead a vegetative existence in the normal mouth, abiding there without causing any appreciable disturb-

* A contemporary review of the literature relating to these subjects has been prepared by Heffron.[601]

ance in the host. The organisms may be virulent or avirulent and
they may sometimes be found as the predominant bacterial species
but, contrary to the older opinion, these so-called normal pneumo-
cocci, although possessed of full virulence, rarely cause pneumonia
in the individual in whom they temporarily dwell. Since the dis-
covery by Sternberg,[1316-8] Pasteur,[1065-6] and other early investiga-
tors that pneumococci virulent for laboratory animals were to be
found in the saliva of healthy persons, many reports have ap-
peared concerning the frequency of the occurrence. Buerger
(1905),[164] by the plate method, detected pneumococci in the
mouths of thirty-nine out of seventy-eight normal persons. Some of
the subjects were assumed to have acquired the organisms in hos-
pital wards and others as a result of pneumonia. In both groups,
the pneumococci persisted for days and even months. In a com-
munication published at the same time, Hiss, Borden, and Knapp,[651]
from their bacteriological study of twenty-two healthy individuals,
concluded that practically every person, at least during the win-
ter season, living under such conditions as exist in New York City,
acts as a host at some time or other and probably at repeated in-
tervals for Pneumococcus. A seasonal effect on the distribution of
pneumococci was suggested by Longcope and Fox[825] who observed
a greater incidence of the organism in normal human beings during
the winter months than during the milder seasons. The authors
stated that during the winter a large percentage of healthy indi-
viduals harbor virulent pneumococci in the buccal cavity, and that
it is almost certain that some persons always have virulent pneu-
mococci in their saliva. More recently, Brown and Anderson
(1932)[152] noted a correlation between the incidence of pneumo-
cocci in the throats of normal persons and periods of inclement
weather.

Park and Williams,[1058] by the mouse inoculation method, found
pneumococci in the sputum of 55 per cent of over two hundred
healthy subjects. McLeod,[880] in reviewing these reports, gave the

more conservative estimate of 20 per cent. Be that as it may, since
the differentiation of the species into the many serological types,
the list of publications which have appeared reveal the widespread
prevalence, wherever investigated, of pneumococci of one type or
another as members of the normal bacterial flora of the mouth.
There is some doubt, however, as to how far down in the respira-
tory tract of healthy individuals pneumococci may be found in a
state of saprophytism. Pneumococcus under favorable conditions
may exchange this benign vegetative existence for a life of malig-
nity for man. According to Hiss and Zinsser—who voiced a gen-
eral opinion—lobar pneumonia is the type of infection most often
caused by Pneumococcus. McLeod differed and expressed the be-
lief that "the most frequent lesion produced by the organism is a
catarrhal condition of the upper respiratory tract involving the
larger bronchi, associated with slight or considerable pyrexia, and
often popularly described as 'influenza.' " The truth of the mat-
ter is of less concern to the bacteriologist than to the clinician. On
the other hand, the fact is incontestable that the great majority
of cases of lobar pneumonia are caused by Pneumococcus. In
addition, Pneumococcus may be the sole causative factor in bron-
chopneumonia, sinusitis, otitis, mastoiditis, meningitis, peritonitis,
nephritis, arthritis, and infections of the eye and other tissues.
Some of the manifestations may be primary in character or
secondary to pneumococcal disease in the lung. In company with
organisms of other bacterial species, Pneumococcus may compli-
cate or intensify the severity of the infection.

Etiology of Pneumonia

PNEUMOCOCCAL TYPES IN LOBAR PNEUMONIA

The predominance of Pneumococcus as the causative agent in
lobar pneumonia with the percentage distribution of other bac-
terial species is seen in the table supplied by Heffron.[601]

Organism found	Number of cases	Per cent
Pneumococcus	3,189	96.1
Streptococcus	94	2.8
Friedländer's bacillus	17	0.5
Influenza bacillus	7	0.2
Staphylococcus	6	0.2
Mixed infections	6	0.2
Total	*3,319*	*100.0*

Heffron in a searching inquiry into the part played by the various serological types in the causation of lobar pneumonia collected all the available data and presented analyses of the figures obtained. In 14,869 cases of the disease reported from many parts of the world, pneumococci of Type I occurred in 32.8 per cent; Type II in 20.6 per cent; Type III in 10.8 per cent; while all organisms listed under Group IV were found in 35.8 per cent of the cases reported. The incidence of pneumococcal types in lobar pneumonia arranged according to geographic distribution, as far as reported, may be seen in the table taken from Heffron.[601]

PERCENTAGE DISTRIBUTION BY SEROLOGICAL TYPE OF 14,869
CASES OF PNEUMOCOCCUS LOBAR PNEUMONIA IN
CERTAIN GEOGRAPHIC AREAS

Geographic group	Total cases	Percentage distribution				
		Type I	*Type II*	*Types I and II together*	*Type III*	*Group IV*
United States, Puerto Rico, and Canada	10,860	31.5	19.2	50.7	11.8	37.5
Europe	3,138	38.7	28.3	67.0	7.0	26.0
Africa	230	19.1	12.2	31.3	2.2	66.5
Far East and Australia	641	41.0	15.4	56.4	6.6	37.0
Total	*14,869*	*32.8*	*20.6*	*53.4*	*10.8*	*35.8*

There occur, of course, local and seasonal variations in the prevalence of pneumococcal types in lobar pneumonia but the foregoing table furnishes a composite picture for the world at large. Since the further division of pneumococci into the present thirty-two serological types, the figures of Sutliff and Finland,[1361] and of Bullowa,[183] as tabulated by Heffron, may be taken as representative of the time and place of observation (Tables A and B).

There is no call to include in this chapter the many further detailed analyses presented by Heffron as to the percentage incidence of pneumococcal types in relation to age and sex of lobar pneumonia patients. It may suffice to include only a summary table showing the collected statistics (Table C).

A. NUMBER AND PERCENTAGE DISTRIBUTION OF CASES OF PNEU-
MOCOCCUS LOBAR PNEUMONIA, DUE TO TYPES I TO XX
INCLUSIVE, IN ADULTS (SUTLIFF AND FINLAND'S
AND BULLOWA'S SERIES)

Type	Sutliff and Finland's cases	Bullowa's cases	Total cases	Per cent
I	268	253	521	30.5
II	142	79	221	12.9
III	121	115	236	13.8
IV	14	65	79	4.6
V	38	60	98	5.7
VI	9	29	38	2.2
VII	37	69	106	6.2
VIII	42	98	140	8.2
IX	14	25	39	2.3
X	19	8	27	1.6
XI	8	6	14	0.8
XII	6	14	20	1.2
XIII	4	8	12	0.7
XIV	14	45	59	3.5
XV	3	1	4	0.2
XVI	1	4	5	0.3
XVII	1	8	9	0.5
XVIII	19	24	43	2.5
XIX	4	16	20	1.2
XX	6	11	17	1.0
Total	*770*	*938*	*1,708*	*100.0*

B. NUMBER AND PERCENTAGE DISTRIBUTION OF 1,000 CASES OF
PNEUMOCOCCUS LOBAR PNEUMONIA, DUE TO TYPES I TO
XXXII INCLUSIVE, IN ADULTS (BULLOWA'S SERIES)

Type	Number	Per cent
I to XX inclusive	770	77.0
XXI	13	1.3
XXII	16	1.6
XXIII	3	0.3
XXIV	3	0.3
XXV	2	0.2
XXVI	–	0.0
XXVII	2	0.2
XXVIII	9	0.9
XXIX	5	0.5
XXX	2	0.2
XXXI	3	0.3
XXXII	4	0.4
Total	*1,000*	*100.0*

C. NUMBER AND PERCENTAGE DISTRIBUTION BY AGE AND SEX OF
TYPES I, II, III, AND GROUP IV CASES OF LOBAR
PNEUMONIA IN ADULTS

PNEUMOCOCCUS	TOTAL CASES	UNDER 50 YEARS				OVER 50 YEARS			
		Men		Women		Men		Women	
		Cases	*Per cent*	*Cases*	*Per cent*	*Cases*	*Per cent*	*Cases*	*Per cent*
Type I	627	462	73.7	73	11.7	83	13.2	9	1.4
Type II	362	236	65.2	44	12.2	76	21.0	6	1.7
Type III	263	124	47.1	42	16.0	70	26.6	27	10.3
Group IV	615	398	64.7	86	14.0	101	16.4	30	4.9
Total	*1,867*	*1,220*	*65.3*	*245*	*13.1*	*330*	*17.7*	*72*	*3.9*

PNEUMOCOCCAL TYPES IN BRONCHOPNEUMONIA

Quoting from Heffron: "The frequency with which the various
types of pneumococci are found in bronchopneumonia approxi-
mately parallels the frequency with which they are carried in the
mouths of normal persons, which suggests that chance occurrence
may play a large part in these infections." So short a time has

elapsed since the serological classification of pneumococci has been enlarged to comprise thirty-two types that the few type analyses in bronchopneumonia can only be looked upon as suggestive. In one of Heffron's tables listing the types of organisms found in this disease in East Africa, Germany, Great Britain, India, and the United States, totalling three hundred cases, it is seen that Type I pneumococci were found in 6.3 per cent, Type II in 4.3 per cent, Type III in 14 per cent, and organisms of the fourth group (type not specified) in 75.3 per cent of all cases reported. There is a marked difference in the reported figures between the incidence of Types I and II combined (10.6 per cent) in bronchopneumonia and their incidence in lobar pneumonia (over 50 per cent).

In observations on the presence of the various pneumococcal types in bronchopneumonia, Sutliff and Finland found that the ten types occurring in a series of 174 cases were, in order of frequency, Types III, VIII, XVIII, X, V, VII, XX, II, XI, and XIV. Additional data are given in the communication of Trask, O'Donovan, Moore, and Beebe.[1417] Studying the relation of pneumococci of the first three types to pneumonia, the latter authors noted that, in patients suffering from Type I or II infections, bronchopneumonia was rare, but with Type III this form of the disease constituted one-third of the affections. Sutliff and Finland reported that 21 per cent of the Type III and 30 per cent of the Type VIII infections studied were cases of bronchopneumonia, whereas only 3 per cent of the Type I and 5 per cent of Type II affections were diagnosed as bronchopneumonia. The early studies at the Hospital of the Rockefeller Institute pointed to extrinsic infection as the cause of lobar pneumonia, whereas the few data available seem to indicate that bronchopneumonia arises more commonly from intrinsic infection or auto-inoculation.

In the case of bronchopneumonia following other affections of the respiratory tract, the infection is largely due to the bacterial species more commonly present in the air passages, among which the streptococci predominate. Pneumococci of the fourth, hetero-

geneous group may account for approximately 50 per cent of the cases. Streptococci of both hemolytic and non-hemolytic varieties have been found in about 31 per cent of the patients studied; the Pfeiffer bacillus and pneumococci of Types I and II were responsible for 3.3 per cent each; while Type III pneumococci appeared two and one-half times as often as did strains of either Type I or II.

PNEUMOCOCCAL TYPES IN PNEUMONIA IN INFANTS AND CHILDREN

The data presented in Heffron's analyses make it appear that, as far as reported, of pneumonia in children under twelve years of age, 9 or 10 per cent of the cases were caused by Type I pneumococci, between 2 and 4 per cent were due to organisms of Type II or III, while in approximately 75 per cent of the cases pneumococci of the remaining types appeared to be the causative organisms. Heffron placed Types I, XIV, VI, V, and VII as the order in which pneumococci are found in the pneumonias of childhood. There appears to be a conspicuous rise in the frequency of pneumococci of the first three types in pneumococcal pneumonia as age increases. Dividing the children studied into one group comprising those under three years of age and a second group in which the ages ranged from three to twelve years, the most marked increase is to be noted in the case of Type I Pneumococcus, which rose from 4.2 per cent in the first group to 20.2 per cent in the second. For pneumococci of Types I, II, and III the corresponding combined incidence was 11.1 and 25.2 per cent respectively in the two age groups. The number of cases examined is too small to allow of accurate statistical deduction, but the trend is significant and points to the greater frequency of organisms of the first three serological types in the higher age groups.

Without burdening the text with too many tables, Heffron's summary may be abridged to read: Among the 826 cases of pneumococcal lobar pneumonia in children of eleven years of age or

under, Type I organisms were found in 22.4 per cent, Type II in 8.1 per cent, and Type III in 5.5 per cent, these three types together accounting for 36.0 per cent of the infections. Sixty-four per cent of the remaining cases were due to pneumococci of the other serological types. Of all the various types, XIV, I, VI, V, and VII were the more prevalent, with IV, IX, XV, and XIX of frequent occurrence.

In pneumonias of the bronchial type the picture is somewhat different. Whereas Type II Pneumococcus showed slightly greater frequency, Type I organisms fell in percentage of occurrence from 22.4 to 9.0, and Types I, II, and III combined showed a decrease of from 36.0 to 26.2 per cent. The respective rates for pneumococci other than those of the first three types were reported to be substantially the same for infants and children and for adults.

To recapitulate the enumeration so far reported of the commonest serological types of Pneumococcus in order of approximate frequency as occurring in infants, children, and adults, the following figures from Heffron are presented:

Lobar pneumonia
    Infants and children . . . . . XIV, I, VI, V, VII
    Adults . . . . I, II, III, VIII, VII, V
Bronchopneumonia
    Infants and children . . . . . VI, XIX, XVIII
    Adults . . . . III, VIII, XVIII, X, V, VII

Serological Types and Fatality-Rates

The factors making for virulence of pneumococci have been discussed in Chapter VI. In addition to the characters of individual strains dependent upon their history and immediate environment, there are variations in invasive power peculiar to the many serological types. The data so far accumulated on the fatality-rates of the different types are not extensive but enough evidence is at hand to warrant some general, if tentative, conclusions. Heffron stated that from available reports the fatality-rates, at least for

lobar pneumonia due to pneumococci of Types I and II, are some-
what lower in Great Britain, and possibly in Norway, Sweden, and
Germany, than in the United States and Canada. For 1,614 civil-
ian cases receiving no serum treatment in the two American coun-
tries, Heffron calculated the death-rate as 25 per cent for Type I
cases and as 41 per cent for cases due to Type II pneumococci,
while of Type III infections from 45 to 60 per cent of the cases
terminated fatally.

FATALITY-RATES OF LOBAR PNEUMONIA DUE TO PNEUMOCOCCI
OF TYPES IV TO XXXII INCLUSIVE

Type	Cases	Deaths	Death-rate per 100
IV	43	13	30.2
V	90	27	30.0
VI	18	8	
VII	97	28	28.8
VIII	67	15	22.4
IX	25	8	
X	24	8	
XI	12	2	
XII	12	5	
XIII	17	4	
XIV	20	2	
XV	4	1	
XVI	6	–	
XVII	3	–	
XVIII	30	10	33.3
XIX	11	4	
XX	10	2	
XXI	2	1	
XXII	–	–	
XXIII	1	1	
XXIV	–	–	
XXV	–	–	
XXVI	1	–	
XXVII	–	–	
XXVIII	–	–	
XXIX	–	–	
XXX	–	–	
XXXI	–	–	
XXXII	–	–	
Total	*493*	*139*	*28.2*

NOTE: Rates are omitted where the numbers are too small to be significant.

Conning the reports published by Bullowa, Park, and Sutliff and Finland, Heffron arranged in tabular form the data representing the fatality-rate in lobar pneumonia caused by pneumococci of types other than the first three.

The above data are too few to permit statistical analysis, yet they are already guiding manufacturers in preparing antipneumococcic serum in accordance with the more prevalent types of pneumococci responsible for lobar pneumonia in this country.

The only report available on the lethal power of the different types of Pneumococcus in bronchopneumonia is that of Sutliff and Finland (1933).[1361] Here again, the data are meager, but there can be no gainsaying the fact that bronchopneumonia caused by pneumococci, with its high death-rate, is a serious affliction to man.

Localized Epidemics of Pneumococcal Infection

The infectiousness of Pneumococcus for individuals living in close association is demonstrated by the occasional outbreaks of acute disease of the respiratory tract. Sinigar (1903)[1292] described an epidemic among the staff and patients of an asylum, but inasmuch as the bacteriological study consisted merely in the microscopic examination of preparations of sputum and from the lungs of fatal cases, one is in the dark as to whether the epidemic was caused by a pneumococcus.

The presence of pneumococci of the various serological types associated with common colds has been too frequently noted to require extended discussion. The results of a study reported by Valentine (1918)[1442] may be cited as typical. From sixty-five cases of upper respiratory infections diagnosed as colds, pneumococci were recovered in forty-three instances. Of the organisms, two belonged to Pneumococcus Type I, two to Type II, four were of the third type, and the remainder fell into the heterogeneous Group IV. A more compact source of material for study was an outbreak of respiratory infection among the inmates of a children's home sheltering more than seven hundred children of both sexes between the

ages of two and twelve years. In investigating the 150 cases occurring among the inmates, Schroder and Cooper (1930)[1250] isolated an organism described by them as extremely infective and which they identified as Type V Pneumococcus. All the cases save one occurred among the boys, who were in frequent and close contact, the sole exception being a girl who had assembled with the boys in a classroom.

A series of twelve cases of pneumonia and two of otitis media developing over a period of four months among eighty-seven boys in an orphanage was caused by Pneumococcus of Type I. Ström,[1344] who studied the epidemic, reported that the infection was more prevalent among the older boys and, furthermore, that of those coming in contact with the affected boys a third of the number were found to be carriers of the same organism. The strains isolated from the patients were more virulent for mice than were those recovered from the carriers.

Infectious Processes Other Than Pneumonia

Pneumococcus, during the pneumonic process, may migrate from the seat of infection in the lung to the vascular system and thereby be distributed throughout the body and create localized foci. Or, without the intermediate pulmonary lesion, the organisms may by direct or indirect routes gain access to vulnerable tissues. While, because of the abundant data, it is possible to apportion the guilt to representatives of the different serological types in lobar and bronchopneumonia, a verdict is not so easily pronounced in non-pulmonary infections of pneumococcal origin. Many of the reports appeared before the diversity of types was recognized, and since that era the comparative infrequency of non-pulmonary infections has supplied too few observations to be of any statistical value.

Meningitis as a complication or sequel of lobar pneumonia had early been reported by Fraenkel,[469] Senger,[1255] Netter,[962] Lancereaux and Besançon,[778] Gamaléia,[498] Meyer,[895] Ortmann,[1037-8] and others, but not until the report of Foà and Bordoni-Uffreduzzi[462]

was any claim advanced that pneumococcal disease of the meninges
could occur as a primary infection. Many references to clinical re-
ports must necessarily be passed over in this review, but Harkavy's
(1928)[589] is cited because from the one patient mentioned Type I
Pneumococcus was isolated from the spinal fluid and in the case of
this patient the administration of specific immune serum effected a
cure.

Endocarditis and pericarditis are not unusual concomitants of
pneumonia, but the condition is nearly always secondary to the
pneumonic process and the same organism may be responsible for
both lesions. Thomas and O'Hara (1920)[1395] described a case of
endocarditis in which Type I Pneumococcus was found in a vegeta-
tion on the tricuspid valve, but the authors disclaimed that the un-
usual site of the infection was due to any special selective affinity
of that pneumococcal type.

Such infections as sinusitis, parotitis, gingivitis, and otitis
may accompany but are rarely independent of lobar pneumonia.
Zaufal[1568-9] was probably the first to report primary infection of
the ear by Pneumococcus. Arthritis is a metastatic manifestation
of pulmonary infection and has been described by Nauwerck,[944-6]
Weichselbaum,[1503-5] and Herzog,[639] but it is improbable that ar-
thritis or nephritis occurs except as a result of pneumococcal in-
fection elsewhere in the body. To the list of non-pulmonary infec-
tions caused by Pneumococcus, Neufeld and Schnitzer added
osteomyelitis and appendicitis (Ungermann), epidydimitis and or-
chitis, cystitis, fibrinous enteritis (Flexner, Curlo), cholecystitis
(Lenharz and others), phlegmon (Robbers), ulcus serpens and
conjunctivitis (Axenfeld and others), erysipelas (v. Leube, and
Reiche and Schomerus), and pyosalpinx and puerperal sepsis
(Jacob).

Of the several specific complications of pneumonia, infections of
the pleura and pericardium are the most common. Endocarditis
and meningitis are grave sequels, while, in addition to the affec-

tions already mentioned, abscesses, retinochoroiditis, and panoph-
thalmia may develop as secondary lesions.

Primary pneumococcal infections of the eye have been described
by Mikaëljan (1931)[902] who, in a limited series of cases, found
pneumococci of Types I and II in panophthalmia, ulcus cornea
serpens, and purulent dacryocystitis, while organisms of Group
IV were isolated from the eye in two cases of purulent conjuncti-
vitis.

In a communication appearing in 1931, Smeall[1293] tabulated the
pneumococcal types encountered in a variety of affections and
stressed the fact that the organisms most commonly found in the
eye and accessory sinuses belonged to Group IV and that those of
Type III and Group IV were present most frequently in acute
otitis media and mastoiditis. It is of course not improbable that a
similar and larger series of cases investigated in another locality
or country might yield different results.

Pneumococcemia

From time to time bacteriologists have alleged that virulent
pneumococci as well as streptococci and other pathogens are to be
found in the circulating blood of healthy persons as well as of
those ill with affections to which the organisms are not related.
The validity of the claims is always highly questionable, and it is
problematical if Pneumococcus invades the circulatory system un-
less a specific focus of infection exists at some point in the body.
Since the discovery by Friedländer[489-90] in 1884 of pneumococci in
the blood of pneumonia patients, their presence has been recog-
nized as a frequent accompaniment of pneumococcal disease indi-
cating a gloomy prognosis. The literature and bedside records are
replete with notes on positive blood cultures taken on pneumonia
patients, but the percentage of positive cultures reported varies
with the nature of the clinical material and the skill of the techni-
cian. Prochaska[1111] claimed that he found pneumococci in the

blood of practically all of forty pneumonia patients; Strouse and Clough (1910)[1347] reported positive results in 56 per cent of the cases occurring in a localized epidemic in which the total mortality was only 20 per cent. Of cultures made by Lyall (1912),[840] 40.5 per cent were positive, and if those taken at the time of crisis or lysis, which were uniformly negative, are deducted, the figure rises to 53 per cent. The fatality-rate for the whole series was 26.2 per cent, while that for the patients having pneumococci in the blood was 50 per cent. The great prognostic value of blood cultures in lobar pneumonia was early pointed out by Avery, Chickering, Cole and Dochez (1917),[36] who reported positive blood cultures in 30.3 per cent of 448 cases. Of the positive cases, 55.8 per cent were fatal, whereas only 8.3 per cent of patients with no pneumococcemia died. The mortality among the patients harboring strains of Types II and III and of Group IV in the blood was above 50 per cent, and all cases in the series with Type III bacteriemia terminated fatally.

In a study made by Sutton and Sevier[1365] at Johns Hopkins Hospital in 1917 the numbers of deaths and of positive blood cultures were practically identical. The series was so small (62) that percentage figures are not of general significance. It was evident, nevertheless, that of the cases observed, positive blood cultures and deaths were far more frequent when the infecting Pneumococcus belonged either to Type I or to atypical Type II. That some organisms of Group IV may be sufficiently invasive to enter the blood stream is shown by the figure of 18.2 per cent of positive blood cultures for cases in which a Group IV pneumococcus was the causative organism.

The correlation between mortality and bacteriemia did not hold in the series reported by McClelland,[874] but pneumococci of Types I and II were responsible for the majority of the positive blood cultures and of the deaths. Here the fatality-rate for Group IV cases was only 4.2—the same as the percentage occurrence of bacteriemia. That pneumococcemia may be present in a large propor-

tion of pneumonia cases is also apparent from the work of Christie in England (1932),[232] who found pneumococci in approximately 57 per cent of the blood samples cultured. Christie emphasized the importance of positive blood cultures as a prognostic measure, since all the fatalities in the series occurred in the positive group, whereas, in the case of the patients who had bacteriemia and recovered, convalescence was prolonged or complicated with empyema.

Heffron discusses this feature of pneumococcal infection in greater detail. For the purposes of the bacteriologist the foregoing discussion may suffice. The presence of active bacteriemia and the development of infected foci in the deeper tissues bears witness of the ability of pneumococci at times to surmount the natural obstacles in the body and to migrate to parts distant from the original site of infection.

Excretion of Pneumococci

From diseased foci pneumococci pass into the sputum in cases of sinusitis, parotitis, gingivitis, and pneumonia; into the spinal fluid in meningitis; into all purulent exudates in pericarditis, empyema, peritonitis, and arthritis. The urine in approximately 38 per cent of the cases of lobar pneumonia studied was found by Mathers (1916)[868] to contain pneumococci. The organisms appeared in the urine usually just before or immediately after crisis, and Mathers advised precautions to avoid the possible danger of passing on the infection, particularly in hospitals, by careless handling of the excreta of pneumonia patients. The intestinal tract is another exit by which pneumococci pass from the infected body. Rutz (1912)[1198] isolated virulent pneumococci from the feces of the majority of patients ill with lobar pneumonia, the organisms appearing as early as the second or third day of the disease. No pneumococci were discovered in the feces of the normal individuals tested.

In studying the distribution of pneumococci throughout the body a word of warning may be said against placing reliance on

the result of cultures taken after the death of the subject. As early as 1905 Norris and Pappenheimer[1018] drew attention to the possible fallacy in making deductions from bacteriological findings at necropsy, since organisms placed in the mouth after death were conveyed to and could be recovered from the lungs by culture in over one-half of the cases in which the post-mortem implantation was made.

The Carrier State

Pneumococci abiding in the mouth and nasopharynx of healthy persons—the chief portal of entrance of Pneumococcus to the body—owe their presence either to direct or indirect contact with other persons harboring the organisms in their oral or nasal cavities, or to previous pneumococcal disease in the same individual. The mere presence of these pathogens in the body is insufficient to incite disease; it is the constitutional factors of the host which determine whether the invaders are to remain as innocuous members of the usual bacterial population or whether they are to abandon their life of saprophytism and act as malignant parasites. In the absence of such depressing conditions as alcoholism, chilling, the exhaustion of fatigue, undernourishment, avitaminosis, or the contributory influence of previous or concomitant disease, Pneumococcus may develop none of its pathogenic potentialities.

The upper respiratory passages of the new-born babe, according to Gundel and Schwarz,[575] are sterile at the time of delivery and may remain so for a few hours after birth. Then on the second or third day pneumococci may appear, the type and number depending upon the quantitative occurrence of similar organisms in the immediate environment, especially in the oral cavity of the mother. The infant as it enters and passes through childhood receives from its fellows and elders contributions from their bacterial flora. In a systematic study of the incidence of pneumococci in the upper respiratory passages of normal persons, largely schoolchildren, Gundel (1933),[569] by repeated tests made every four weeks

for a year on over one hundred subjects, found that while the type of Pneumococcus usually proved to be the same in successive tests on a given individual, in many cases there were rapid and frequent changes in the types present. The relative infrequency of organisms of the first three types, so often observed in the mouth flora of healthy persons, is also to be noted in Gundel's report. Type I pneumococci occurred in 0.8 per cent, Type II in 0.4 per cent, Type III in 6.7 per cent, and organisms of Group IV (Gundel's X) were present in 60 per cent of the individuals tested successively throughout the year.

In a continuation of the study, Gundel and Okura,[573] with even more painstaking methods, investigated the occurrence of pneumococci of more than one serological type in the same subject. Thirty-eight per cent of the individuals of the series tested carried organisms of two or more types, and the frequency of occurrence was much greater among boys than among girls. The appearance of new types was attributed by the authors to infection from without or possibly to the development of a type which had been suppressed by the dominance of the first type found. Gundel and Okura did not agree with Neufeld and Etinger-Tulczinska[984] that infection of the nasal or buccal mucous membrane may lead to specific immunity against the infecting type without eliciting any apparent or at least definite morbid symptoms, since repeated serological tests failed to reveal any specific antibodies for the types carried for protracted periods. The explanation that the non-invasiveness of pneumococci existing in the normal mouth could be due to the fact that the organisms there undergo dissociation into rough and, therefore, avirulent forms was opposed by Gundel and Schwarz,[575] who encountered no such variants in their investigations. This opinion was further supported by the results of a slightly earlier series of virulence tests on pneumococci isolated from normal and pneumonic sputums, which would seem to show that virulent strains exhibit few if any signs of variation *in vivo*. Gundel and Wasu[578] concluded that the vegetative pneumo-

cocci were not culpable in causing any ensuing pneumonia but that
the infection always came from without.

The disappearance of members of the first three types during
convalescence and the subsequent appearance of organisms of a
heterologous group was early established by Dochez and Avery
(1915).[319] The authors stated that although pneumococci occur in
the mouths of 60 per cent of normal individuals, the organisms are
readily distinguishable from the highly parasitic types of pneumo-
cocci responsible for the severe forms of lobar pneumonia—a con-
vincing proof that infection in that disease is, in the majority of
instances, not autogenic in nature, but is derived from some ex-
traneous source. Dochez and Avery found further that in a high
percentage of instances healthy persons intimately associated with
cases of lobar pneumonia harbor the disease-producing types of
pneumococci. In every such instance the strain isolated from the
normal subject was found to correspond in type with that of the
infected individual with whom the healthy person had come in con-
tact. In conclusion, the authors stated that the existence of the
carrier state among healthy persons and among those recently re-
covered from pneumonia establishes a basis for understanding the
mechanism by means of which lobar pneumonia spreads and main-
tains its high incidence from year to year.

The relative infrequency of pneumococci of the first three types
in the mouths of healthy persons of varying ages who gave no his-
tory of contact with pneumonia was also observed by Meyer
(1920).[899] Among one hundred normal individuals, no strains of
Type I or II (excepting an atypical II), and only three strains
of Type III pneumococci were found. Group IV organisms were
present in seventeen instances. In the sputum of fifty tuberculous
patients Lyall[841] found pneumococci in twenty cases. Excluding
one patient with a history of pneumonia in the previous year,
pneumococci of Type I occurred once, of Type II none were found,
of Type III three subjects harbored the organism, while pneu-

mococci of Group IV accounted for 75 per cent of the positive cultures.

The studies reported by Rosenau, Felton, and Atwater,[1157] and by Powell, Atwater, and Felton[1106] in 1926 yielded results which, while differing in some respects from those of Dochez and Avery, confirm their thesis and throw additional light on the carrier problem. Using a thoroughgoing technique for the isolation of pneumococci from the mouth and throat, the Boston authors isolated pneumococci of Type I from four times as many subjects in contact with cases of lobar pneumonia as normal persons not thus exposed. Type III organisms were recovered in twice as many instances for every one hundred cases in the first group as in the second, but there was no appreciable difference in the incidence of Type II pneumococci. Strains belonging to Group IV occurred in 83.5 per cent of the former and in 69.3 per cent of the latter subjects. The differences in the percentages representing the incidence of pneumococci of the first three types, as reported by the Boston and by the New York observers, may have been due to the broader inclusion by the Boston workers of persons in the near neighborhood of pneumonia patients.

The rarity of pneumococci of Types I, II, and III in persons far removed from centers in which pneumonia is endemic has been reported by Milam and Smillie (1931).[903] In the isolated tropical island, St. John, of the United States Virgin Islands, the pneumococci found in the nasopharyngeal flora of normal persons were avirulent, and representatives of the first three types were seldom present. Even city dwellers in a colder climate may enjoy comparative freedom from the more common disease-producing types of pneumococci. In an extended investigation, embracing monthly examinations over a period from two months to three and one-half years, of 105 children and adults living in New York City, Webster and Hughes (1931)[1495] obtained pneumococci at one time or another from the nasal passages or the throat of 80 per cent of the

persons studied. Of the 500 strains isolated, 97 per cent proved to
be serologically specific. The organisms formed smooth colonies
and were for the most part avirulent for mice. Pneumococci of
Types I and II were obtained from one and two individuals respec-
tively on one occasion only. Type III organisms were encountered
in nine subjects, Type VIII in nine, Types XVI and XVIII in
three persons for varying periods in each case; and avirulent,
atypical strains were isolated from thirteen persons on single and
scattered occasions. The presence of the last-named organisms was
considered by the authors as having no association with any type-
transformation *in vivo*.

The strains isolated in successive cultures from a given carrier
were, with rare exceptions, of the same serological type and similar
in colony morphology, virulence for mice, and other biological
characters. The persons observed differed consistently with respect
to the occurrence of pneumococci. Some were Pneumococcus-free,
some were transient carriers, some periodic, and some chronic car-
riers, and evidence was presented in the communication that these
differences were due to variations in host-resistance. The incidence
of pneumococci in all the individuals included in the study under-
went seasonal fluctuations corresponding to changes in the preva-
lence of coryza and sore throats in the same persons, an observa-
tion reported by Longcope and Fox[825] in 1905.

Further data on the variety of pneumococci to be found in the
nose and throat flora of individuals selected only for the non-
existence of pneumonic disease among them, are furnished by the
report of Hoyle (1932)[662] who, like Webster and Hughes, carried
out bacteriological observations on normal persons over periods of
one, one and a half, and two years. Of the forty subjects, on one
occasion—and the only one—Type I Pneumococcus appeared dur-
ing an acute attack of coryza, the patient developing lobar pneu-
monia three or four days later. Type II organisms were never de-
tected. Type III pneumococci were isolated on four occasions, once
late in a cold, in one case persisting for fourteen days in the mouth

flora of a carrier of Type IV, once in an individual free from other
pathogenic bacteria, and once during an attack of mild bronchitis
subsequent to a cold. The other pneumococci encountered belonged
to the heterogeneous group.

Another recent report is that of Brown and Anderson,[152] who
found no strains of the first three types, but only those of the re-
maining types—all of low virulence for mice—from a small series
of normal subjects. Christie (1932),[232] in making cultures from
the throats of nurses in a Glasgow hospital, isolated pneumococci
from eleven of twelve nurses in the pneumonia ward but none from
nurses in the control wards. Of the organisms found in the nurses
exposed to pneumonia, Christie demonstrated Type I pneumococci
four times and Type II seven times. Whether the organisms found
corresponded to the types prevalent among the pneumonia pa-
tients was not stated in the communication.

The classification of carriers of Pneumococcus based on re-
peated bacteriological examinations extending over a year or more
as drawn up by Webster and Hughes was slightly modified by
Bliss, McClaskey, and Long (1934).[131] After a year's study of
young adults, the authors divided the subjects into non-carriers
and chronic carriers, the latter group including those persons who
intermittently exhibited pneumococci in the throat. While the so-
called intermittent carriers might or might not yield positive cul-
tures on repeated examination, the cultures when positive were
consistently of the same type of Pneumococcus in any given case,
indicating to the authors a chronic condition with constant bac-
teriological findings only as to type. Furthermore, the authors
considered that their demonstration added evidence in favor of the
stability of pneumococcal types in the human body. From the
foregoing discussion it would seem to be more logical to designate
healthy individuals who harbor pneumococci in the nose and throat
for short periods of time as temporary carriers and those in whom
the organisms persist for longer periods as chronic carriers. A
subdivision of the latter into continuous and intermittent carriers

would define more accurately the condition and the possible menace of the chronic class.

Summary

A summary of the main facts brought out in the foregoing discussion may be presented thus:

1. The great majority of cases of lobar pneumonia (*circa* 96 per cent) are caused by Pneumococcus.

2. Pneumococci of Types I and II are accountable for approximately one-half of the cases of lobar pneumonia in all countries from which records are available, except Africa.

3. The various serological types found in lobar pneumonia, as far as at present known, are, in order of frequency, I, III, II, VIII, VII, V, IV, XIV, XVIII. In children, pneumococci of the first three types are responsible for the disease in only 36 per cent of the cases, with Types XIV, I, VI, V, VII, IV, IX, XV, and XIX occurring most frequently in the order given.

4. The fatality-rates of pneumococcal lobar pneumonia have been reported as 40 to 60 per cent for Type III cases, 41 for Type II, 25 for Type I, with Types XVIII, VII, and VIII next in order of lethal power.

5. In bronchopneumonia, the types responsible for infection in order of incidence are III, VIII, XVIII, X, V, VII, XX, II, XI, and XIV. The data are tentative pending further information.

6. Lobar pneumonia, although usually endemic, may, as is the case of other bacterial infections of the respiratory tract, appear in localized outbreaks, which are not the peculiar manifestation of any particular type of Pneumococcus.

7. As accompaniments or sequels of lobar pneumonia, infections by the pneumococcus causing the primary lesion may arise in various locations in the body, or

8. Primary lesions incited by Pneumococcus may develop in the eye, nasal, buccal, and aural cavities and adnexa, the meninges, and other tissues.

9. Healthy persons may harbor pneumococci of one or more types in the mouth and throat with no harm to themselves.

10. The carrier state may be due a) to pneumococci implanted by transference from other persons or b) to organisms arising from pneumococcal disease within the individual.

11. The organisms implanted by transference are largely of the heterogeneous types formerly classified as Group IV, except in the event that the donor is suffering or recovering from an attack of pneumonia due to the predominant types. The pneumococci that are autogenous in origin are of the type causing the pneumonia.

12. The carrier state may be brief (transient), prolonged (chronic), or sporadic (chronically intermittent).

CHEMICAL CONSTITUENTS OF PNEUMOCOCCUS

The preparation and properties of the protein and carbohydrate fractions of the pneumococcal cell, discussed in relation to their chemical constitution and immunological activities.

THE chemical complexity of pneumococci and the immunological significance of their constituents were never appreciated, or even suspected, until Heidelberger and Avery[606] announced the results of their study of the composition of these remarkable cells. Like many other bacteria, Pneumococcus had been neglected by chemists. Such information as we had was either based on assumption or had come from chance or collateral observations by bacteriologists. Protein, of course, was present, protein combined with phosphorus being a part of all living cells. Then fats or lipids revealed their presence by the fatty acids they yielded on autolysis of the cell. Inorganic salts were elements necessary for its vital functions, although there was no detailed knowledge of their kind or quantity. The capsule, so distinctive of the species, had been believed to consist of mucin or some allied substance. It was, therefore, the systematic study of Heidelberger and Avery (1923) which disclosed the presence of the peculiar carbohydrates that give to strains of each type their special serological characters, that spell virulence or, by their absence, lack of virulence, and that determine the exact or specific immunological response the cocci call forth. These studies, moreover, rendered a greater service in revealing a new biological principle, that is, the action of sugars as antigens per se or in orienting the antigenic stimulus of their conjugated protein, and in their exquisite action as haptens in the presence of homologous immune bodies.

Carbohydrates had always been disregarded as possessing any

immunizing properties—it was thought to be the proteins of the cells which performed this function—but now it seems that, in the case of Pneumococcus at least, the protein is merely the vehicle carrying the sugar that decides the immunological character of the saccharide-protein complex of the cell.

WORK OF EARLIER INVESTIGATORS

Before discussing the newer discoveries it may be permissible to recount briefly the observations of earlier investigators in order that their bearing on these questions may be borne in mind. The literature before 1923 is notable for its lack of reports of detailed or systematic investigations on the chemistry of Pneumococcus. Here and there one finds communications dealing with a few of the components of the cocci, some fragmentary, some pointing mostly by inference to the existence of proteins, fats, or carbohydrates. The first account was that of Friedländer[487] in 1883. His interest in the peculiar capsular material caused him to carry out a few simple tests to determine its nature. It was soluble in alkali, but insoluble in acetic or mineral acids, in alcohol, ether, or chloroform, and these properties led Friedländer to believe that the capsule was composed of mucin or some related substance.

While it was a foregone conclusion that the cell contained protein, no direct evidence had been offered proving its existence. The affinity of the cellular material for aniline dyes was presumptive evidence, as was the increase in amino nitrogen during autolysis. The work of Rosenow[1169] and of Avery and Cullen[38, 41] proved that intracellular protein must be the source of the smaller nitrogenous molecules arising in the natural self-digestion of the cell. So it was with the fats; only by their cleavage products were they recognized.

Prior to 1917, none of the constituents of Pneumococcus had been isolated or subjected to chemical study. It was in that year that Dochez and Avery[321] discovered in cell-free filtrates of broth

cultures of Pneumococcus of Types I, II, and III, in human blood
serum, in urine during the course of lobar pneumonia* and, at
times, in the blood of experimentally infected animals, a soluble
substance that gave a specific precipitate with antipneumococcic
serum of the homologous type. According to the authors, the sub-
stance was present in cultures when the organisms were growing at
maximal rate and undergoing little or no cell death. Consequently
its presence was not dependent upon cell disintegration, but repre-
sented the extrusion of bacterial substance by the living organism.
The substance was readily soluble in water, was not destroyed by
boiling, was not digested by trypsin or urease, did not dialyze
through parchment, and was precipitable by acetone, alcohol,
ether, and colloidal iron. The substance was, of course, the now
well-known soluble specific substance which has so broadened our
conception of pneumococcal immunity.

From a careful reading of Perlzweig's two papers (1921)[1078-9]
there seems to be no doubt that he was dealing with the same sub-
stance but in impure state. From solutions of Pneumococcus in
bile-salts, by Rowland's anhydrous sodium sulfate method, and by
alcohol, Perlzweig precipitated an antigenic substance which he
considered was a nucleoprotein. However, on autolyzing the whole
organism or digesting it with proteolytic enzymes, the antigen was
found to be unimpaired. It was not injured by boiling for five min-
utes in neutral or slightly acid solution, but was destroyed by boil-
ing in alkaline solution. The substance could be recovered from
autolysates or digestion mixtures by extraction with alcohol in a
concentration of 70 to 85 per cent, but it was not soluble in 95 to
99 per cent alcohol. Perlzweig,[1079] and Perlzweig and Steffen,[1081]
described the antigen as being soluble in neutral, acid, and alka-
line aqueous solutions but not in lipid solvents and, when tested
antigenically on white mice, it apparently possessed the complete
immunizing properties of the original pneumococcus. Because of

* The finding recalls the observation of Preisz[1108] (see Chapter II) who dem-
onstrated the presence of this substance in the blood of pneumonia patients, but
who thought it was mucin.

its physical characters and the dependence of its heat stability on the hydrogen ion concentration of the substrate, the authors suggested that this antigen might be closely related to the antineuritic water-soluble B vitamin.

In another communication (1925), Perlzweig and Keefer[1080] reported a method for purifying the antigen but gave no details of its immunological properties. From massive cultures in Huntoon's medium, after heat-killing and filtration, the antigenic substance was separated from non-antigenic material by ultrafiltration through collodion. Further purification was effected by precipitation at the isoelectric point (pH 4.1) with 0.1N acetic acid-sodium acetate buffer mixture. The precipitate was dissolved in water with the addition of a small amount of 0.1N sodium hydroxide. The preparation contained 20 milligrams of nitrogen per 100 cubic centimeters (based on the volume of the original solution), which might indicate that it was made up, in part at least, of the nitrogen-containing specific carbohydrate of Type I Pneumococcus, the organism used as source material. Since Perlzweig and Keefer gave no other details, it is impossible to judge the purity of their antigen, but Perlzweig and Steffen,[1081] in a 1923 report, considered that it existed in a loose chemical or physical union with protein. It is to be presumed, therefore, that the authors had not isolated the polysaccharide in pure form, but rather the carbohydrate-protein compound from which some of the protein had been removed.

At about the same time, Zinsser and Parker,[1583] continuing the study made by Zinsser[1575] on tuberculin, described an antigen obtained from Pneumococcus and other bacteria which was analogous to, if not identical with, the substance isolated by Dochez and Avery.[321] These "residues" or "residue antigens" were prepared in the following manner: Pneumococci were grown on agar, removed from the medium and dried, then shaken with salt solution at a pH of 9.0 to 9.4, and immediately used or neutralized and stored. The extracts were centrifuged, passed through a Berkefeld

filter, and precipitated with acetic acid in the cold. After separation of the precipitate (phosphoprotein or nucleoprotein), acid was added until no more precipitate came out of solution when the precipitate was discarded. After filtration, the solution was acidified and boiled for three to five minutes, then again filtered and neutralized. The filtrate was the residue solution. It became turbid on the addition of ten volumes of alcohol, and the precipitate contained the antigenic substance, although the authors suggested that it might have been mechanically thrown down.

The residue gave a specific precipitate with homologous antiserum but no complement fixation with equine antipneumococcic serum, nor did it stimulate any immune-body production in animals injected with it. The substance, however, excited a positive reaction when injected intradermally into normal guinea pigs and, to a lesser degree, in tuberculous pigs. Its resistance to autoclaving and to boiling in alkaline solutions would seem to establish a close relationship to the soluble specific substance described by Dochez and Avery.

Two years later, Zinsser with Tamiya[1584] extended the study of pneumococcal residue and pneumococcal protein. Some rabbits were immunized with agar-grown pneumococci washed with 2 per cent formalin in saline solution, and other rabbits with Berkefeld filtrates of pneumococci dissolved in minimal amounts of bile. The serum from these animals was then tested for precipitins against the two antigens. In the discussion of the experimental results Zinsser and Tamiya expressed the opinion that the antigens consisted of two substances: the one obtained by treating pneumococci with weak alkali, the other by fractioning the extract by acid precipitation. The one was a nucleoprotein independently antigenic, inducing antibodies which reacted only with itself and not with the residue; the other was the residue material incapable of inducing any kind of antibody response but capable of reacting with antibodies formed by the injection of the whole bacteria. The two substances, therefore, although in a crude state, corresponded in their immu-

nological reactions to the protein and carbohydrate fractions of Avery and Heidelberger.

FIRST DESCRIPTION OF THE PNEUMOCOCCAL CARBOHYDRATE

It was, however, Heidelberger and Avery who first gave definite and detailed descriptions of the carbohydrate fraction, or soluble specific substance of pneumococci. No microorganism, except perhaps the tubercle bacillus, has since that time been subjected to such thorough chemical investigation as Pneumococcus. The studies of Heidelberger and Avery and their colleagues and those coming from other laboratories have supplied a rationale for the varied immunological and pathological behavior of pneumococci and established a chemical basis for understanding many of the diverse phenomena of immunity.

In their first communication, Heidelberger and Avery (1923)[606] described the isolation of the soluble specific substance of Type II Pneumococcus, which they concluded consisted mainly of a carbohydrate, which appeared to be a polysaccharide built up of glucose molecules and which, in a dilution as high as 1 to 3,000,000, gave a specific precipitin reaction with homologous immune serum. The method of its preparation was simple. It consisted in the concentration of an eight-day autolyzed broth culture of Type II Pneumococcus, precipitation with alcohol, repeated resolution and precipitation, then a careful series of fractional precipitations with alcohol or acetone after acidification with acetic acid and, finally, repeated fractional precipitation with ammonium sulfate and dialysis of aqueous solutions of the active fractions. The product gave no biuret reaction and no precipitate with phosphotungstic acid. It contained a trace of phosphorus but no sulfur and, because 1.2 per cent of nitrogen was found to be present, the authors forebore to make any claim for the purity of the preparation.

During the next few years there came from the laboratories of the Hospital of the Rockefeller Institute a series of papers under the authorship of Avery and Heidelberger and their associates,

Goebel, Morgan, and Neill, which contained descriptions of refinements in the methods of preparing these polysaccharides and of their physical, chemical, and immunological properties. Because of their importance the data are presented here in considerable detail; and in order that the reader may be offered a full, connected, and authentic account of the results of these basic studies, the liberty is taken of quoting from and paraphrasing Heidelberger's[604-5] comprehensive discussions in 1927 of the chemical nature of the antigenic substances of Pneumococcus.

The type II Pneumococcus was first studied and the fractionation and purification of the specific substance were followed at each step by means of the precipitin test. . . . As the purification proceeded the material isolated took on more and more the properties of a polysaccharide, so that it became evident that a sugar derivative was at least the carrier of whatever might be the true specific substance itself. Attempts were made to separate this hypothetical substance from the polysaccharide by precipitation with basic lead acetate, uranyl nitrate, or safranine, by adsorption with alumina and recovery from this, and even by specific precipitation with a large quantity of Type II antibody solution (prepared by Felton's method), and recovery of the specific substance from the immune precipitate, but these failed to effect a significant change in properties, even when pneumococci themselves were used as starting material instead of the broth culture. Attempts at a separation by means of certain carbohydrate-splitting enzymes also failed since the sugar derivative proved resistant to this type of hydrolysis. Moreover, when exposed to the action of 1:1 hydrochloric acid in the cold, the substance diminished in specific activity only after reducing sugars began to appear, so that the specific substance and the polysaccharide, if not identical, appeared at least to be very closely associated. On hydrolysis the specific product yielded about 70 per cent of reducing sugars consisting mainly of glucose, as shown by the isolation of glucosazone and the formation of saccharic acid on oxidation. Other possible constituents remained unidentified.

Marked differences were found between the soluble specific substance of Type III Pneumococcus and the corresponding derivative of Type II. The former proved to be the soluble salt of an in-

soluble acid, far stronger than the Type II substance, and capable of being thrown out of solution by an excess of strong hydrochloric acid. This property was of great use, not only in separating the specific substance from accompanying glycogen or erythrodextrin, but also in effecting rapid purification without the use of ammonium sulfate and with fewer fractionations by alcohol. Successive lots agreed closely in their physical and chemical properties, indicating a much more definite entity than the Type II product. The Type III soluble specific substance was thus isolated as a nitrogen-free polysaccharide. No further purification could be realized either by precipitation with barium hydroxide in excess, or by adsorption on highly active alumina. The yield of this soluble specific substance was greatly increased by adding glucose to the broth.

In the case of Type I Pneumococcus, the amount of the soluble specific substance present in the culture fluid was relatively smaller than in the case of the Type II and III organisms and therefore more alcohol was required for its precipitation from the culture concentrate. Other modifications were necessitated by the insolubility of the substance at its isoelectric point (about pH 4), and advantage could be taken of its ability to form a precipitate with barium hydroxide in excess. The specific substance was reprecipitated by alcohol in the presence of hydrochloric acid and dialyzed. Being a weak base, it precipitated as the excess of hydrochloric acid was removed. The Type I soluble specific substance also appeared to be a carbohydrate, but differed in the lower percentage of sugar liberated on hydrolysis and, what is more distinctive, in containing nitrogen as an apparently essential component.

Heidelberger, Goebel, and Avery[611] arranged a comparison of the distinguishing characters of the soluble specific substances of Types I, II, and III, and included that of the Type B Friedländer bacillus (to be mentioned later) in the table which is reproduced on the following page.

The aldobionic acid, $C_{11}H_{19}O_{10}COOH$, isolated from the hydro-

lytic products of the specific polysaccharide of Type III Pneumococcus was shown to be a compound of glucuronic acid and glucose, combined in glucosidic linkage through the aldehyde group of glucuronic acid and one of the hydroxyl groups of glucose. The polysaccharides from the three types of pneumococci contained no sulfur or phosphorus and differed from the starch-glycogen group in giving no color with iodine and in their resistance to the usual carbohydrate-splitting enzymes. These polysaccharides, therefore, would appear to represent a new type of carbohydrate.

SOLUBLE SPECIFIC SUBSTANCES OF THE THREE FIXED
ANTIGENIC TYPES OF PNEUMOCOCCUS AND OF
FRIEDLÄNDER BACILLUS (TYPE B)

Type	$[a]_D$	Acid equivalent	C	H	N	Reducing sugars on hydrolysis		Highest dilution giving precipitate with homologous immune serum
			Per cent	Per cent	Per cent	Calculated as glucose		
I	+300°		43.3*	5.8	5.0†	28	(Galacturonic acid) (Amino sugar derivative)	1:6,000,000
II	+74°	1250	45.8	6.4	0.0	70	Glucose	1:5,000,000
III	−33°	340	42.7	5.3	0.0	75	Aldobionic acid, glucose	1:6,000,000
Friedländer bacillus B	+100°	685	44.6	6.1	0.0	73	Glucose	1:2,000,000‡

* Theory for $(C_6H_{10}O_5)_n$, C, 44.4 per cent; H, 6.2 per cent.
† Amino N, 2.5 per cent.
‡ Rabbit antiserum.
Note: From a determination of its diffusion coefficient, Babers and Goebel[61] calculated a molecular weight of 118,000 for Pneumococcus III specific polysaccharide, but Heidelberger and Kendall[618] later found that this was an accidental value, related only to the salt concentration at which the diffusion was carried out.

Heidelberger and Avery doubted if any of the specific substances isolated at that time (1927) represented a definite chemi-

cal compound. The three types of Pneumococcus chosen for study had, however, when grown on the same medium, yielded three distinct carbohydrates, and successive preparations of each specific substance had been quite uniform whatever methods were employed in the process of purification and whether the preparation was derived from pneumococci themselves or from autolyzed broth cultures. Furthermore, the only one of these substances investigated in detail appeared to differ in structure from that of any other known non-nitrogenous polysaccharide. It was thought by Avery and Heidelberger that these and other considerations based on the data obtained warranted the belief that the three polysaccharides isolated represented the actual specific substances, stripped of at least a large portion of accompanying impurities, and that the substances did not merely represent inert material carrying an extremely minute amount of the true specific compounds.

The studies of Heidelberger, Goebel, and Avery also included an investigation of the protein portion of the pneumococcal cell. Heidelberger, in the reviews already cited,[604-5] discussed this phase of the work as follows:

When these microbes (Pneumococci) are dissolved, either with the aid of bile, or by repeated freezing and thawing, the resulting solution yields a precipitate of so-called "nucleoprotein" on acidification with acetic acid. While probably a mixture consisting largely of nucleoprotein and mucoid, it still possesses immunological properties which differ sharply from those of the soluble specific substance. In the first place, the protein is antigenic, while the soluble specific substance, though reacting specifically with antibodies to the highest degree, is non-antigenic and unable by itself to stimulate the production of antibodies when injected into animals. Moreover, the protein isolated from the three fixed types of Pneumococcus, or from a strain of the heterogeneous group IV, appears serologically the same as that from any of the other types. Thus this portion of the pneumococcus protein is not type-specific, like the soluble specific substance, but is, rather, species-specific.

Saito[1213] also prepared nucleoprotein from Type II pneumococci which precipitated with both Types I and II serums and

with which active immunity could be evoked against Type II but not against Type I Pneumococcus. Saito admitted that the preparation still contained traces of the soluble specific substance.

An apparent relationship between pneumococcal antibodies and both the protein and non-protein fractions of Gonococcus has been reported by Boor and Miller (1931).[138] The protein substance—nucleoprotein according to the authors—in a dilution of 1 to 1,000 gave definite precipitation with Types I and II antipneumococcic serum and in as high as a 1 to 10,000 dilution with Type III serum. The non-protein fraction was even more potent, reacting positively, and always more strongly with Type III serum, in ten to one hundred times the maximal dilution of the protein fraction.

Boor and Miller observed cross reactions between the gonococcal antiserum and the C Fraction of Tillett and Francis and there is good reason to believe that the non-protein fraction obtained from Gonococcus and the C Fraction of Pneumococcus are similar if not identical. The results, because of their seeming biological importance, deserve further study. There is a factor, however, which should not be overlooked and that is the participation of agar in serological reactions, as demonstrated by Sordelli and Mayer[1306] in the case of other organisms grown on agar media.

Stull (1929)[1349] furnished figures for some of the chemical constituents of Type III Pneumococcus.* A virulent strain of the organism was grown in beef-infusion peptone glucose broth for sixteen to eighteen hours and centrifuged; then the sediment was washed in distilled water, and dried at 55°. Analyses of the material gave the following results expressed in percentage amounts: Volatile matter at 105°—4.77; ash—6.48 to 8.64; nitrogen—13.00 to 13.32; acid-insoluble nitrogen and acid-soluble nitrogen

* In this connection there should be mentioned the analyses of Leineweber, Kautsky, and Famulener,[799] which were taken to indicate the comparative uniformity of pneumococci of the four types in the amount of nitrogen, and therefore of protein contained.

—each 0.2; ammonia nitrogen—1.0; total nitrogen of bases—5.5; total nitrogen in filtrate from bases—8.6; phosphorus—2.92 to 2.94; chlorine—0.71; and sulfur—0.32. An absolute alcohol extract of the dried cocci contained 6.5 per cent nitrogen and one per cent phosphorus and was claimed to be entirely free from protein. Water extraction removed material of high phosphorus content, probably of the nature of nucleoprotein, as well as the soluble specific substance. Stull's ether, alcohol, acetone, 0.1N acetic acid, and 0.1N hydrochloric acid extracts all failed to give precipitates with Type I, II, and III antipneumococcic serums. Only the dried cocci, the water extract, and the soluble specific substance precipitated with Type III serum, and here the action was specific.

It was Avery and Heidelberger's conception that, since Pneumococcus is an encapsulated organism, "The ectoplasmic layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type-specific substance. On the other hand, the endoplasm, or somatic substance, consists largely of protein, which is species- and not type-specific. This protein is possessed in common by all pneumococci, while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends." It was also the authors' idea that, since this specifically reactive substance was found to be non-antigenic when separated from the other cellular constituents and was capable of inciting antibody formation only in the form in which it is present in the intact cell, it might be concluded that in the latter instance it existed not merely as free carbohydrate but also in combination with some other substance which conferred upon it specific antigenic properties.

A tabular representation of some of the immunological functions of intact pneumococci and of their principal constituents is taken

from the review[605] which has been drawn upon so freely in the present discussion.*

PNEUMOCOCCUS AND CELL CONSTITUENTS		ANTIBODIES DEMONSTRABLE IN SERUM						
Material used for immunization	Effective antigen	Agglutinins	Pre-cipitins		Comple-ment fixation		Speci-ficity	
			S	P	S*	P	Type	Species
Intact cells (SP)†	(SP)	+	+	−	+	−	+	−
Carbohydrate S‡	None	−	−	−	−	−	−	−
Protein P§.	P	−	−	+	−	+	−	+
Solutions, extracts containing free S and free P	P	−	−	+	−	+	−	+
Suspension of intact cells and dis-sociated cell constituents (SP), free S, free P	(SP), P	+	+	+	+	+	+	+

* = Free S, as antigen, does not fix complement with immune horse serum; is active with immune rabbit serum.
† (SP) = Carbohydrate and protein, combined antigen of cell.
‡ S = Free carbohydrate, the soluble specific substance of cell.
§ P = Free protein of cell.

Heidelberger continued:

It was evident that morphological dissolution of pneumococci is accompanied by antigenic dissociation, for sera prepared from filtered solutions of disintegrated cells free of formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria. The injection of suspensions of pneumococci into animals induces the formation of antibodies against S [carbohydrate] alone or against both S and P [protein] separately, depending upon whether or not these suspensions contain only intact cells or a mixture of both intact and dissolved cell bodies. Since pneumococci readily undergo autolysis and dissolution, suspensions and broth cultures of these organisms almost invariably contain not only formed elements, but also more or less of dissociated cell constituents in solution. Therefore, use of suspensions of pneumococci con-

* Many of the experimental data from which the table was compiled are to be found in the original papers by Avery and Heidelberger; Heidelberger and Goebel; Heidelberger, Goebel, and Avery; Heidelberger and Avery; Avery, Heidelberger, and Goebel; Avery and Morgan; and Avery and Neill.

taining both intact cells and the soluble products of cell disintegration
yields on immunization not only type-specific antibodies but antibodies
reacting with the protein substance which is common to all pneumo-
cocci. While the former generally predominate it is the presence of this
protein antibody with its broader zone of activity which is responsible
for the confusing cross-immunity reactions occasionally encountered in
supposedly type-specific sera and which has in some instances led work-
ers to deny the existence of three distinct antigenic types of pneumo-
cocci. That the two sets of antibodies involved are separate and distinct
is shown by absorption tests; the antiprotein reaction bodies in such
sera can be removed by absorption with the protein of a heterologous
type without diminishing the titer of specific agglutinins for the homolo-
gous culture or the precipitins for the specific polysaccharide of the
corresponding type. . . .

While it had been generally assumed that only the proteins and their
derivatives provided the innumerable opportunities for isomerism and
subtle changes requisite for the substances exhibiting the phenomena of
specificity, the discovery of carbohydrates with specific properties is not
so surprising as might appear on first thought. When one considers the
number of asymmetric carbon atoms in the hexoses and pentoses, the
different points of attachment of the lactone bridge, the possibility of
α and β-glucosidic unions at various positions in the molecule, and the
addition of sugar acids, the analogs of amino acids, to the large number
of sugars theoretically capable of entering into the composition of such
polysaccharides, it becomes clear that perhaps only among the carbo-
hydrates could another sufficiently large and protean group of sub-
stances be found to afford the possibility of specific manifestations.

The evidence of the lack of immunizing properties on the part of
these specific carbohydrates shown in the table on page 250 was
largely supplied by the experiments of Avery and Morgan.[54] Their
attempts to immunize rabbits by subcutaneous and intravenous in-
jections of the protein-free polysaccharides of pneumococci in con-
siderable amounts and in repeated doses invariably failed to stimu-
late the production of any demonstrable antibodies in the serum
of rabbits so treated. The isolated protein, on the contrary, was
antigenic, but the antibodies it induced reacted with the nucleo-
protein fraction of both homologous and heterologous types. In

addition, these antiprotein serums did not agglutinate type-specific strains of Pneumococcus or react with the type-specific carbohydrate derived from them. This fact points to the capsular polysaccharide of the cell as the bearer of the type-specific determinant.

As a complement to these observations, Avery and Neill[59] added that intact pneumococci, possessing specific antigenic powers unimpaired by cultural or other procedures, gave rise to agglutinins for the homologous type and to precipitins for the type-specific carbohydrate derived from them. When, however, the cell was disrupted, the soluble cell-free constituents in the absence of formed elements failed to stimulate the formation of type-specific antibodies, but did induce the formation of antibodies reactive with pneumococcal protein regardless of the type from which the latter was derived. The data pointed to the dissociation of the carbohydrate and protein fractions during the process of cell dissolution with the subsequent change in the type-specific action of the constituents, or, put in another way, to the loss of antigenic function of the polysaccharide portion of the cell when freed from its conjugated protein.

ISOLATION OF CARBOHYDRATE FRACTIONS

From the results reported by Schiemann and Caspar,[1228] it would appear that they had isolated two carbohydrate fractions from Type II Pneumococcus. One of the protein-free substances was soluble in alcohol, gave specific precipitation with homologous serum, and was antigenic in that it immunized mice. The second fraction also gave specific precipitation but was insoluble in alcohol. The preparations were made by boiling sodium taurocholate solutions of pneumococci with acetic acid. Both substances and the one obtained from a Type III strain were evidently free from protein, since the authors could detect no nitrogen in the preparations, and yet contrary to the results of Perlzweig and Steffen and of Avery and Morgan, the non-nitrogenous fractions possessed antigenic power since they produced immunity in mice. Schiemann

and Caspar noted a difference in the microscopic appearance of the precipitates produced by the carbohydrates when added to immune horse and rabbit serum. The difference in the behavior of these two kinds of serum is manifested in other reactions with pneumococci and their constituents or products. The protein fractions as a rule failed to establish protection in mice, although one such preparation from Type II Pneumococcus did induce resistance of a low order to pneumococci of both Types I and II.

Jungeblut (1927)[698] had quite a different idea concerning the chemical nature of the antigenic components of the pneumococcal cell. Pneumococci of the first three types were extracted with 95 per cent alcohol and the filtrates of these extracts were tested for specific flocculation by a modified Dujarric de la Rivière method. Antipneumococcic serums of Types I, II, and III gave flocculation of varying intensity with homologous alcoholic antigens. The reactions were species-specific and also type-specific to a high degree. From an examination of the experimental facts, and after a consideration of the data presented prior to this study, one is not inclined to go all the way with Jungeblut in concluding that this serological reaction depended upon the presence of bacterial lipids in the antigens. Remembering the activity of minute amounts of specific carbohydrates in bringing down precipitins from immune serum, one feels that emphasis should be laid on Jungeblut's comment that the method of preparing these alcoholic antigens did not preclude the possibility that "certain impurities of protein or carbohydrate character may have been carried over into the extract."

Wadsworth and Brown[1467] reported the antigenic action of the ether-soluble fraction of Type I pneumococci. The preparation, admittedly impure, was active in binding complement in the presence of antipneumococcic serum of all types but failed to stimulate the formation of agglutinins, precipitins, protective or complement-fixing antibodies in rabbits.

In 1928, Saito and Ulrich,[1214] following the lead of Heidelberger

and Avery, prepared what appeared to be a protein-free carbo-
hydrate from Pneumococcus II and which produced specific pro-
tection in mice. After dissolving the sediment from a twenty-four-
hour serum-broth culture in sodium taurocholate, the authors, by a
method much the same as that of Schiemann and Caspar, removed
the precipitate coming down on the addition of acetic acid, and
then separated the specific precipitable substance by alcohol. A
concentrated watery solution of the material was then treated with
a large amount of normal sodium hydroxide and the resulting sedi-
ment discarded. Upon the addition of alcohol to the clear fluid a
precipitate was obtained, which in a slightly acidified solution
failed to give any positive protein tests, had a dextro-rotatory
power of about $+30°$ as compared with that of $+74°$ for the
Type II carbohydrate of Heidelberger, and which on boiling with
hydrochloric acid yielded reducing substances.

The carbohydrate preparation of Saito and Ulrich was strictly
type-specific, giving protection to mice against a Type II culture
but no protection against a Type III culture. It seems fair to as-
sume that their carbohydrate preparation was not so pure as the
corresponding preparation of Heidelberger and Avery since its
dextro-rotatory power was less ($+30°$ against $+74°$) and also
since it contained 0.61 per cent of nitrogen.

Schiemann[1226] employed the preparations made by Saito and
Ulrich in an attempt to immunize rabbits, and attributed his fail-
ure to the administration of too great dosage. In no instance was
it possible to produce agglutinins, precipitins, or protective anti-
bodies. He succeeded, however, in immunizing mice and in demon-
strating protective substances in their blood.

Schiemann, Loewenthal, and Hackenthal[1231] continued the study
of pneumococcal carbohydrates and retested the conclusions of
Perlzweig and Steffen.[1081] From acid and alcohol precipitates of
dissolved Type I pneumococci extracts were made with methyl and
ethyl alcohol. The authors reported that the carbohydrate frac-
tion contained the larger proportion of the immunizing substance

and that the alcoholic extracts showed varying specific immunizing properties, which they believed might be due to the presence of small amounts of the type-specific carbohydrate carried over by the alcohol. It was found, furthermore, that watery solutions of the carbohydrate still produced immunity in dilutions which no longer gave precipitation.

The protein-free substance of Schiemann and his associates, purified by repeated alcohol precipitation in alkaline solution, showed +264°10' angle of refraction with polarized light (compare +300° for Heidelberger and Avery's Type I polysaccharide), and with hydrochloric or nitric acids at 80° yielded no reducing sugars. The authors noticed the characteristic differences in the appearance of the precipitates obtained with specific immune horse and rabbit serum—the former appearing sooner and being coarser, while the latter were fine and formed a transparent membrane. The purified preparation actively immunized mice in doses of 0.001 to 0.0001 milligrams but failed to do so when injected in amounts greater than 0.01, or less than 0.00001 milligrams, and therefore acted in a narrower zone than the less pure fractions prepared by the method of Schiemann and Caspar.[1228]

In contrast to the immunizing action of the carbohydrate fractions of Pneumococcus in mice, as reported by Perlzweig and Steffen, Schiemann and Caspar, Saito and Ulrich, and Schiemann, Loewenthal and Hackenthal, the polysaccharides from Type I, II, and III pneumococci were devoid of any power to sensitize guinea pigs to these specific carbohydrates. When single initial doses of ten, twenty, and fifty milligrams of the preparations were injected the animals failed to react when given a shocking dose of one to ten milligrams twenty-one days later. This same shocking dose, however, produced rapid and fatal anaphylactic shock when injected into guinea pigs passively sensitized with the precipitating serum of rabbits immunized with pneumococci of the homologous type. The experiment afforded evidence of a striking difference, therefore, between the antigenic and haptenic action of preparations of

specific polysaccharides. Here again there was manifested that strange difference between immune rabbit and horse serum, the latter failing to sensitize the guinea pigs to the carbohydrate. Both serums precipitate the homologous carbohydrate, but the horse serum fails to bind complement in the presence of capsular polysaccharide as well as being incapable of rendering guinea pigs hypersensitive.

ISOLATION OF C FRACTION

The existence of another and quite different carbohydrate constituent of Pneumococcus was demonstrated by Tillett and Francis.[1409] The authors used as source material a degraded, non-type-specific R strain of Pneumococcus. This particular culture was chosen in order to minimize the presence of type-specific carbohydrate. The organisms centrifuged from a full-grown broth culture were suspended in normal salt solution, the cells were frozen and thawed until dissolution was effected, and then, after the addition of acetic acid, the solution was boiled for eight to ten minutes. After removal of the heavy coagulum, acidulation and boiling were repeated in order to remove all acid- and heat-precipitable material. The final water-clear supernatant fluid contained this new substance which Tillett and Francis called "Fraction C."*

The C Fraction, although it is probably a nitrogenous sugar, is chemically distinct from both the type-specific capsular carbohydrate and the somatic nucleoprotein. It exhibits no type-specificity, but yields a precipitate with serum of individuals ill with lobar pneumonia. Following crisis the reaction is no longer demonstrable. Furthermore, the precipitation of the pneumococcal Fraction C is not limited to the serum of individuals infected with Pneumococcus, since definite reactions can be obtained in streptococcal and staphylococcal infections and in acute rheumatic fever.

In another communication appearing in the same year, Tillett

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* Tillett and Francis remarked on the similarity of this substance to Lancefield's "Fraction C" which she had obtained from hemolytic streptococci.

with Goebel and Avery[1410] continued the study of the newly iso-
lated fraction. The fraction was further purified by making the
solution (the water-clear supernatant fluid described above) alka-
line with sodium hydroxide and by precipitation with alcohol. The
material was dissolved in water and again reprecipitated from
faintly acid solution by alcohol. The procedure was repeated sev-
eral times. The final precipitate was washed with alcohol and ether
and freed from chlorides and was soluble in water but insoluble in
organic solvents. The substance in solution showed a specific rota-
tion of $+25.0°$, contained 5.07 per cent of nitrogen, and yielded
30.0 per cent of reducing sugars on hydrolysis. Unlike the type-
specific polysaccharide of Type I Pneumococcus it contained no
amino nitrogen, but like other pneumococcal carbohydrates was
protein-free. Tillett, Goebel, and Avery also made similar prepara-
tions from S strains of Type II and Type III pneumococci.

The C Fraction was non-toxic for mice in amounts up to one
milligram, it produced no purpura, and an intravenous injection
of three cubic centimeters of a concentrated solution elicited no
symptoms in rabbits. Three series of seven daily injections of one
cubic centimeter of the concentrate, with weekly rests, failed to
produce in rabbits any precipitins for the C substance. When
mixed with antipneumococcic horse serum of Types I, II, and III
the somatic carbohydrate precipitated each of the three, thus ex-
hibiting a broad species coverage but no type-specificity. Tillett,
Goebel, and Avery concluded from chemical studies and animal ex-
periments that Fraction C was a common constituent of all pneu-
mococci, and that it was distinct from the specific polysaccharide
and the so-called nucleoprotein.

The isolation of the C Fraction was also accomplished by Hei-
delberger and Kendall (1931)[617] during an investigation of the
polysaccharides of Type (not Group) IV Pneumococcus. They also
obtained the C substance from Type I and Type III pneumococci.
The preparations showed a higher optical rotation, higher nitro-
gen, and in two cases a higher reducing-sugar content on hydroly-

sis than reported by Tillett, Goebel, and Avery who, it should be remembered, prepared their material from rough pneumococci. An analysis of the C Fraction showed that it contained 4 per cent of phosphorus, which appeared to be firmly bound in organic combination, thus making the C Fraction the first phosphorus-containing specific polysaccharide to be encountered.

RELATION OF THE PNEUMOCOCCAL CARBOHYDRATE TO CARBO-
HYDRATES ISOLATED FROM OTHER ORGANISMS

In addition to the C Fraction, Heidelberger and Kendall isolated from autolyzed cultures of Type IV Pneumococcus a type-specific carbohydrate differing markedly from those of Type I, II, and III pneumococci, and representing a kind of substance hitherto not observed among specific polysaccharides. The authors also obtained a chemically similar carbohydrate without specific function. These substances were far more difficult to separate from accompanying protein degradation products than the specific polysaccharides of Type I, II, and III pneumococci. Because of the difficulty of separation, the preparations probably contained more or less of the accompanying specific carbohydrates, but were in sufficiently pure state to reveal separate identities.

The serologically inactive fraction had the lowest optical rotation and the highest carbon content of the three, was the weakest acid, the least soluble in alcohol or acetic acid, and differed from the specific Type IV carbohydrate and the C Fraction in yielding on hydrolysis crystals with the optical rotation of glucosamine. The Type IV specific substance, on the other hand, differed from the others in being the poorest in nitrogen and the richest in reducing sugars on hydrolysis, and in occupying an intermediate position as regards optical rotation, carbon content, and acidity. The species-specific C polysaccharide was highest in optical rotation and in total and amino nitrogen, and the poorest in reducing sugars yielded on hydrolysis, in carbon content, and in its propor-

tion of acetylated nitrogen. It differed from the fully acetylated Type IV substance in being broken down by nitrous acid, and differed from all other known specific polysaccharides of Pneumococcus in containing phosphorus.

From this comparative study of the various specific carbohydrates of Pneumococcus it would seem that these substances fall into two sharply defined groups: on the one hand, the Type II and Type III type-specific polysaccharides, which are nitrogen-free, and on the other, the Type I, Type IV, and C Fractions which contain nitrogen. The two last-named examples in this group, with their content of acetylated nitrogen, are more closely related to chitin than is the Type I substance. In the nitrogenous group, the Type I substance differs sharply from the others in its high optical rotation, its pronounced amphoteric character, its insolubility at the isoelectric point, its freedom from acetyl groups, and in its high proportion of nitrogen susceptible to attack by nitrous acid. The C Fraction differs from the other members of both groups in its phosphorus content, but resembles the Type I substance in that its specificity is destroyed by nitrous acid; it is somewhat similar to the Type IV substance in that a part of the nitrogen is acetylated. The Type IV soluble specific substance, on the other hand, differs from the Type I polysaccharide and the C Fraction in containing only acetylated nitrogen and in yielding as high a percentage of reducing sugars on hydrolysis as do the nitrogen-free Type II and Type III capsular polysaccharides.

The study of the Type IV specific polysaccharide has brought to light a carbohydrate of a new type among those with specific properties. It has, moreover, again been shown that in the closely related pneumococcal types thus far studied, each polysaccharide responsible for type-specificity is different from the others in structure, composition, and properties. The study has also demonstrated the presence in Pneumococcus of a serologically inactive polysaccharide closely related to chitin. This observation points

toward the cause of the still existing uncertainty as to whether chitin is a constituent of the bacterial cell wall.*

In addition to these specific pneumococcal polysaccharides, Heidelberger, Goebel, and Avery[611-2] isolated from the B strain of the Friedländer bacillus a carbohydrate closely resembling that of Type II Pneumococcus, the similarity extending even to precipitation with Type II antipneumococcic serum. But, although there was great constancy in the properties of these two substances, the absorption of agglutinins and precipitins was not reciprocal with the two organisms. It was believed that the cross-relationship was due to the occurrence in the specific polysaccharides of both microorganisms of the same or similar chemical grouping. Other strains of the bacillus showed no such reciprocal relations, nor was there any such relation between the E strains of the Friedländer group and Type I and III pneumococci.†

Heidelberger, in the review already quoted, argued that if the fact that bacteria possess mutual absorption capacity be accepted as the criterion of their antigenic identity, then the failure of the Friedländer bacilli to exhibit this property might be taken as further evidence of the lack of identity of the substances involved. The discussion continued:

However, granted a chemical difference between the two specific substances, it becomes necessary to account for their marked immunological similarity. In the absence of further evidence as to the structural relations of the two polysaccharides it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. The essential similarity in molecular grouping would then determine the immunological similarity of the two substances. In the case of Pneumococcus it has been shown that the polysaccharides by themselves are not antigenic, and it is believed that

* The foregoing paragraphs are based on the discussion in the paper by Heidelberger and Kendall.[617]

† In this connection it may be mentioned that in the next year Julianelle[684] classified, by their serological reactions, strains of *Encapsulatus pneumoniae* (Friedländer's bacillus) into three types, A, B, C, and one group X.

It may interest the reader to know that in 1935 Yen and Kurotchkin[1559] by means of electrolysis were able to isolate the specific carbohydrate of the Friedländer bacillus, apparently free from protein and in a highly antigenic state.

they become antigenic only when attached to some other substance, possibly the protein of the cell. The type specific character of the antigenic response, however, is dependent almost entirely upon the nature of the polysaccharide and not upon the substance to which it is attached. Therefore, since the specific carbohydrate of the Friedländer bacillus (type B) and that of Type II Pneumococcus exhibit similar chemical properties the antigenic response to each may also be similar even though the proteins or other substances with which they are combined are quite dissimilar.

In 1929, Heidelberger, Avery, and Goebel[608] described the isolation of a soluble specific substance from gum arabic (gum acacia). From this gum, which would appear to have no biological relationship to Pneumococcus, by partial hydrolysis a carbohydrate was obtained that was comparable to the specific pneumococcal polysaccharide in its precipitating activity with both Type II and Type III serum. The fraction on hydrolysis yielded galactose and two more complex sugar acids, one of which was later shown by Heidelberger and Kendall[617] to be aldobionic acid and glucuronogalactose analogous to the compounds isolated from the specific polysaccharide of Type III Pneumococcus.

Another manifestation of heterogenetic specificity was that of the encapsulated strains of *Escherichia coli*, studied by Barnes and Wight.[87] The organism, isolated from a mouse during a type-determination test on pneumonic sputum, was agglutinated by Type I antipneumococcic horse serum, but not by a similar serum produced in a rabbit. The serum from rabbits immunized with this colon bacillus agglutinated the homologous organism and precipitated the soluble specific substance, but failed to cause agglutination of Type I pneumococci or to precipitate Type I pneumococcal polysaccharide. In this case the connection was somewhat analogous to that between Type II Pneumococcus and the later Type B Friedländer bacillus of Julianelle.

Zozaya[1588] published an account of the cross and quite heterologous serological reaction with antipneumococcic serum of dextran, the synthetic polysaccharide produced from saccharose by *Leuco-*

nostoc mesenteroides. With serum produced in rabbits in response to injections of typical and rough strains of Types I and II Pneumococcus, dextran in a dilution of 1 to 5,000 gave precipitates with both anti-S and anti-R serums in low dilutions (1 to 2 to 1 to 16), showing a somewhat stronger action with the latter. When the serums were first absorbed with the type-specific polysaccharide or the C Fraction of Tillett and Francis, and then mixed with dextran, the serum precipitated to the same degree as before absorption. The outcome of the experiment would argue for the existence of a distinct antibody produced by the active group of the specific polysaccharide, which is similar to the active group of the dextran polysaccharide.

Since the discovery of the soluble specific substance in Pneumococcus by Dochez and Avery in 1917, similar complex carbohydrates have been demonstrated as components of several bacterial species. Toenniessen[1413] was the first to isolate the nitrogen-free polysaccharide from Friedländer's bacillus, which was later studied by Kramár,[753] but failed to connect it with the immunological behavior of the organism. A related substance was recovered from a strain of the same organism by Mueller, Smith, and Litarczek,[937] from yeast by Mueller and Tomcsik,[938] and from an encapsulated colon bacillus and *Bacillus aerogenes* strains by Tomcsik.[1414] A specifically precipitating, non-nitrogenous carbohydrate has been isolated from tubercle bacilli by Laidlaw and Dudley,[771] and a similar polysaccharide as well as a specifically reacting substance was derived by Mueller[936] from the same bacilli by fractionation with alcohol.

In a preliminary report of a recent study, Kulp and Borden[764] described the successful immunization of mice against Type I Pneumococcus by vaccination with a living culture of *Alkaligenes viscosus*. The authors suggested that there might be an antigenic relationship between the capsular material of these two organisms, but they had obtained no confirmation of this possibility. There is no doubt that other bacterial species contain analogous polysac-

charides which may be found to possess similar chemical and immunological relationships to those of Pneumococcus but their existence in no way detracts from the soundness of our present serological classification of pneumococci. Tempting as it is, further discussion of the immunological significance of the carbohydrates from sources other than Pneumococcus would not be pertinent to the present subject.

FUNCTION OF SUGARS IN DETERMINING ANTIGENIC SPECIFICITY AND CONJUGATED PROTEINS

The newly discovered and highly important function of sugars in determining the antigenic specificity of conjugated proteins was studied by Goebel, Avery, and Tillett. The two first-named authors,[521] aiming to gain more exact information concerning this specific action of carbohydrates, set about building two isomeric carbohydrate-protein compounds. They first synthesized p-aminophenol β-glucoside and p-aminophenol β-galactoside and then coupled these hexosides with the globulin from horse serum. The two protein-sugar complexes thus obtained differed only in the carbohydrate radical of each and in the spatial configuration of the H and OH groups by a single carbon atom. In a second paper Avery and Goebel,[44] after coupling the diazophenol glucosides to crystalline egg albumin in addition to serum globulin, studied the immunological behavior of the preparations. When two chemically different carbohydrate derivatives were bound to the same protein, the newly formed antigens exhibited distinct immunological specificity. When the same carbohydrate radical was conjugated with two chemically different and serologically distinct proteins, both of the sugar-proteins thus formed acquired a common serological specificity. Therefore, simple differences in the molecular configuration of the two isomers—glucose and galactose—sufficed to orient specificity when the corresponding glucosides were coupled to the same protein. The unconjugated glucosides, although themselves not precipitable in immune serum, specifically inhibited the

reaction between the sugar-protein and its homologous antibody. Furthermore, the sugar derivatives unattached to protein exhibited some of the properties of carbohydrate haptens; they were non-antigenic in that they incited no antibody production, but were specifically reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized glucoside.

Tillett with Avery and Goebel[1407] tested these artificial, conjugated carbohydrate-proteins for other evidence of antigenic action by means of experiments in active and passive anaphylaxis. The experiments demonstrated the capacity of the synthesized sugar-proteins to produce hypersensitiveness. The fact that guinea pigs, passively sensitized with antigluco-globulin serum, or actively sensitized with gluco-globulin, could be subsequently shocked with gluco-albumin, and since the same specific relations held in the production of anaphylaxis with galacto-proteins, it was evident that the antigen-antibody specificity in these instances was directly dependent upon the carbohydrate fraction of the antigenic compounds.

In addition to the new specificity which the carbohydrate radical conferred upon the conjugated proteins, the uncombined glucosides by themselves also exerted a definite influence on the reactivity of sensitized animals. The injection of the glucosides into guinea pigs sensitized with the homologous gluco-protein immediately before the introduction of the toxigenic sugar-protein, completely but only temporarily protected the animals from shock. Tillett, Avery, and Goebel found, also, that anaphylactic shock could be induced by uncombined globulin in guinea pigs passively sensitized with either antigluco-globulin serum or antigalacto-globulin serum, thus demonstrating that the reactions elicited by globulin alone were dependent upon the common protein present in the antigens, and were manifestations only of species-specificity.

Continuing the investigations, Goebel and Avery[522] prepared the p-amino and p-nitromonobenzyl ethers of the polysaccharide of

Type III Pneumococcus, which they succeeded in coupling with se-
rum globulin. Avery and Goebel[45] then reported the important and
fundamental observation that this artificial, specific carbohydrate-
protein complex was able to immunize rabbits against infection
with virulent Type III pneumococci, and that the serum of rabbits
thus immunized contained type-specific antibodies which precipi-
tated the Type III capsular polysaccharide, agglutinated Type
III pneumococci, and specifically protected mice against Type III
infection. From the results it would seem that the antipneumo-
coccic response produced by an antigen known to contain but a
single component of the pneumococcal cell indicates the unity of
the antibodies participating in the type-specific reactions of pre-
cipitation, agglutination, and protection, and relates the speci-
ficity of these antibodies to that of the capsular polysaccharide as
the reactive part of the antigenic molecule.

ISOLATION OF "A SUBSTANCE"

What appeared to be a new element among the specific polysac-
charides was introduced by Enders[358] in 1930. In autolytic prod-
ucts of Type I Pneumococcus a substance was found, thought to
be other than the soluble specific substance, which reacted spe-
cifically with immune serum as determined by the precipitin reac-
tion or, *in vivo*, by the anaphylactic behavior of appropriately
sensitized guinea pigs. Enders prepared autolysates by incubating
for seventy-two hours the phenolized, centrifuged sediment from
twenty-four-hour dextrose broth cultures of Type I pneumococci.
He obtained the nucleoprotein by precipitating with dilute acetic
acid the solution derived by dissolving pneumococci with bile ac-
cording to the method of Avery and Heidelberger, while the spe-
cific carbohydrate was prepared from pneumococci grown in dex-
trose hormone broth according to the methods described by
Heidelberger and his associates.

What appeared to Enders prepared a "normal" antipneumococcic serum by inject-
ing rabbits intravenously with cultures of Type I Pneumococcus

grown in broth containing rabbits' blood and killed by the addition of formalin. He obtained an immune rabbit serum by treating the animals intravenously with repeated injections of the autolysate and of the killed hormone broth-rabbit blood cultures and this serum was designated "Anti-A" serum. In addition, to other rabbits Enders gave injections of formalinized saline suspensions of agar-grown pneumococci. When the Anti-A serum was tested against SSS* no precipitate was formed, but with an autolysate from smooth Type I Pneumococcus the serum was definitely precipitating, and the reaction was type-specific. Furthermore, both the Type I autolysate and the supernatant fluid from the acetic acid precipitation of the autolysate gave precipitates with Anti-A serum but none with antipneumococcic serum prepared from rough strains.

By the method devised by Ward, Enders then added to his "normal" serum specific carbohydrate until precipitation was complete. The centrifuged supernatant fluid no longer gave any visible precipitation with SSS but, when the homologous autolysate was added, an abundant, flocculent precipitate was observed. Inasmuch as the SSS had apparently exhausted its specific antibody from the immune serum, the appearance of a precipitate on the addition of autolysate indicated to Enders the presence in the autolysate of a precipitinogen other than the specific polysaccharide and, accordingly, to this hypothetical substance he gave the name, "A Substance."

There are two other characters of the A substance of Enders which should be mentioned. One was instability when exposed to heat. Boiled for one-half hour at pH 9 its effectiveness as a precipitating antigen was reduced at least one thousandfold, while the soluble specific substance under similar treatment remained unchanged. When, however, the reaction was adjusted to pH 4 with 10 per cent acetic acid, a solution containing the A substance

* For convenience, the soluble specific substance or type-specific polysaccharides of Pneumococcus will hereafter be frequently referred to as SSS.

could be boiled for the same period of time without losing its activity. In both acid and alkaline solution, autoclaving for one hour at fifteen pounds pressure practically destroyed its effectiveness. The second character was its resistance to pepsin-hydrochloric acid mixture. The preparation probably resisted also the digestive action of trypsin, but the presence of alkali in such a substrate, by itself, tended to destroy the substance and therefore interfered with any accurate determination of its digestibility.

Enders[359] extended the investigation to embrace the carbohydrates of Type II and III pneumococci and, in addition to precipitative and anaphylactic methods, employed the agglutination and agglutinin-absorption reactions for a further comparison of his new antigen with the specific polysaccharide. He reported that type-specific agglutination of Type I, II, and III pneumococci occurred in homologous antiserum from both rabbit and horse to approximately the same titer after the antibody reacting with the purified specific carbohydrate had been removed. This fact suggested that in pneumococci there exists a type-specific agglutinogen which was to be distinguished from the specific carbohydrate. A side comment of Enders was that the presence of a specific agglutinogen in Pneumococcus Type II unrelated to the specific carbohydrate would account for the failure of this organism to remove agglutinins from *B. Friedländeri* Type B antiserum, and would add additional evidence pointing to dissimilarity of the A substance and the soluble specific substance as well as to the lack of serological identity of Pneumococcus II and Friedländer bacilli of the B type.

An effect analogous to that described by Enders was reported by Ward[1483] in 1932. In a five-day broth culture of Type III Pneumococcus he found a type-specific substance with a powerful antibactericidal action. In comparison with the soluble specific carbohydrate, its precipitating action was far greater, requiring a much larger amount of antipneumococcic serum for neutralization. A similar substance, but in higher concentration, was also found in

the filtrate of a lung obtained at necropsy from a Type III pneumonia patient. Tested by his whole-blood method, Ward found
that a specimen of Type III convalescent blood, though comparatively weak in anticarbohydrate antibody, was better able to neutralize the broth filtrate and lung filtrate than a corresponding
mixture of normal blood and antiserum. Two other specimens of
Type III convalescent blood neutralized the Type III broth filtrate. Ward attempted no isolation of this precipitinogen but
concluded: "The possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate—
perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate
itself—was forced on the author as the most likely explanation."

Evidence which might be taken as indicating the existence of the
A substance of Enders, or of some similar principle in Pneumococcus, is to be found in the work of Sabin (1931),[1204] who, like
Enders, but independently,* attempted to exhaust potent antipneumococcic horse serum of protective antibodies by preliminary
saturation with homologous SSS and found that in spite of this
absorptive treatment the serum still contained an appreciable
quantity of the specific protective substance. There appeared to be
a lack of proportionality between the quantity of SSS used for
precipitation and the amount of protective antibody left in the
supernatant fluid. In the reaction some of the protective antibody
was evidently lost, since the sum of the precipitated and free antibody represented only from 20 to 50 per cent of the total. This
large loss might be accounted for if an excess of specific carbohydrate were added since it would exert the inhibiting action observed by Felton. The same results as those obtained in the *in vitro*
experiments were noted in *in vivo* tests in the rabbit.†

Sabin concluded that in antipneumococcic serum there was a

* Enders' paper was received for publication in May, 1930, and published in
August of the same year, while Sabin's communication was accepted in September, 1930, and appeared in January, 1931.

† Incidentally, an observation of interest was that in the combination of SSS
with its homologous precipitin there occurred a phenomenon similar to the

type-specific antibody which was not neutralized by SSS and which was apparently distinct from the anticarbohydrate precipitin. Reasoning that this non-precipitable antibody could not have been evolved in response to any antigenic stimulus from the soluble specific substance, Sabin sought an answer to the question, "Is there another type-specific antigen in the Pneumococcus in addition to the SSS?" After adding to Type I antipneumococcic serum the required amount of SSS for complete precipitation, he centrifuged the mixture after water-bath and ice-box incubation, and treated portions of the supernatant liquid with a 50 and 100 per cent excess of SSS, with a saline suspension of heat-killed Type I pneumococci, and with a similar suspension of heat-killed Type II pneumococci. The experiment showed that the residual protective antibody in the supernatant fluid was not neutralized by SSS or by absorption with Type II pneumococci, but was definitely absorbed with the homologous Type I pneumococci. Sabin assumed, therefore, that the neutralization was specific, and unless SSS in the organism was capable of neutralizing the antibody which SSS in solution could not, some other substance in Pneumococcus must be the responsible agent. This proviso, however, is all important in this connection. There is no proof that the SSS as prepared by Sobotka for Sabin, even though by his tests it was practically identical with the soluble specific substance of Heidelberger and Avery, was identical with the specific polysaccharide as it exists in the pneumococcal cell.

In 1931, Wadsworth and Brown[1466] reported the isolation of a carbohydrate which appeared to be analogous to the soluble specific substance and which yet more closely resembled the A substance of Enders. The original source material was a virulent Type I strain, and the method consisted in removing the organisms from fifteen-and-one-half hour broth cultures by means of the

Danysz effect in the combination of toxin and antitoxin. More SSS was required for complete precipitation when the total quantity was added at once, than when it was added in fractions of the total on successive days. This effect had also been observed by Heidelberger and Kendall in their studies on the precipitin reaction.

supercentrifuge and washing the sediment in distilled water. The supernatant fluid and washings were evaporated over the free flame to small volume and then used for the isolation of the specific carbohydrate by the method of Heidelberger and Avery. The preparation contained about 5 to 6 per cent of nitrogen. Its solution gave negative biuret and xanthoproteic tests, a positive Molisch test, did not reduce Fehling's solution until after hydrolysis with hydrochloric acid, and was precipitated by phosphotungstic acid. The material was more readily soluble in water than the polysaccharide of Heidelberger and Avery.

When tested against antipneumococcic serum, the dissimilarity between the substance prepared by Wadsworth and Brown and the soluble specific substance of Heidelberger and Avery and its similarity to the A substance of Enders became evident. It precipitated specifically in Type I serum in a dilution of 1 to 6,000,000, but in a dilution of 1 to 600,000 it gave an immediate ring reaction with Type I serum which had been completely absorbed with a highly purified preparation of the soluble specific substance. Wadsworth and Brown did not test the supernatant fluid from the serum precipitated with SSS for protective antibody.

Again differing in properties from the Heidelberger and Avery polysaccharide, the substance induced immunity in mice, as demonstrated by the protection test. The immunizing effect was type-specific. The substance fixed complement in the presence of Type I antipneumococcic rabbit serum and while it caused no reaction when injected intravenously in a dose of one milligram into a normal guinea pig it evoked fatal anaphylactic shock in guinea pigs passively sensitized not only with Type I antipneumococcic rabbit serum but with the same serum after removal of precipitin by absorption with the soluble specific substance. Wadsworth and Brown concluded that the substance they had isolated from the pneumococcal cell corresponded to that of Schiemann and his coworkers, and to the A substance of Enders, but was distinct from the soluble specific substance of Heidelberger and Avery.

In another communication, Wadsworth and Brown[1468] reported on chemical and immunological studies of carbohydrate fractions separated from pneumococci of the first three types and from an atypical strain as well. The cultures used were a virulent Type I Neufeld strain, a strain each of virulent Types II and III, and an attenuated Type I Pneumococcus. The carbohydrates resembled in character and action the substance described by Schiemann and his colleagues. The preparations differed in essential respects from the soluble specific substance of Heidelberger and Avery, so Wadsworth and Brown, for the purpose of distinguishing their substance from SSS, gave to it the somewhat ambiguous name, "Cellular Carbohydrate."

In a 1 to 300 dilution none of the preparations gave biuret, Millon's, or xanthoproteic tests; the Molisch test was positive and, after hydrolysis, Fehling's solution was reduced. The results of the micro-analysis of the "cellular carbohydrates" are shown in the original table taken from the paper by Wadsworth and Brown. The figures represent minimums and maximums. One specimen from each of the type preparations gave a trace of sulfur; the remainder gave none.

The apparent presence of phosphorus in organic combination in all these preparations revealed a distinct and, to the authors, possibly an important chemical difference between the antigenic and non-antigenic carbohydrate. The presence of amino nitrogen and of only small amounts of phosphorus further distinguished the cellular carbohydrates from the C Fraction of Tillett and Francis. Unlike the SSS of Types II and III, the cellular carbohydrates of these types contained nitrogen and phosphorus, although nitrogen had never been found in analogous preparations by Heidelberger and phosphorus had been shown by Heidelberger and Kendall to be a constituent only of the C Fraction. These unusual results and the wide variations in the analytical figures lead to the surmise that these preparations of cellular carbohydrates were impure.

MICRO-ANALYSIS OF THE CELLULAR CARBOHYDRATES OF THE PNEUMOCOCCUS

Type of cellular carbohydrate of Pneumococcus	Preparation number	Sulfur	Phosphorus*	Total nitrogen†	Amino nitrogen‡	Ash	Water
			Per cent	Per cent	Per cent	Per cent	Per cent
I	6		0.43	4.28		0.90	13.52
I	9		0.39	4.37	2.14	0.43	9.51
I	12	Trace	0.35	4.01	2.03	0.85	13.73
I	13		0.77	4.59	1.91	2.40	10.18
Atypical I	1			7.66		12.65	9.12
Atypical I	3		5.36	8.92		1.64	8.61
Atypical I	4		6.54	5.99		2.05	7.87
II	1		1.72	2.03		0.62	6.54
II	2	Trace	1.86	2.83		0.08	6.75
II	3		1.75	3.20		2.09	9.68
III	1		4.58	6.11		3.10	7.75
III	2		2.56	3.75		1.08	9.99
III	3	Trace	2.06	3.41		1.76	9.95

* Elek's modification of Pregl's micro method.
† Pregl micro-Kjeldahl method.
‡ Van Slyke method.

By precipitating a concentrated neutral solution of the Type I cellular substance with hydrochloric acid, Wadsworth and Brown, just as Heidelberger, Goebel, and Avery had done, obtained an acid-insoluble and an acid-soluble fraction.

When tested with homologous antipneumococcic serum, both of the fractions in a dilution of 1 to 6,000,000 gave precipitation. When a Type I rabbit serum, which had been absorbed separately with each fraction, was tested with the other, the acid-soluble fraction failed to precipitate serum absorbed by either, while the acid-insoluble fraction precipitated to a slight degree with the serum absorbed with the acid-soluble portion. Unfortunately, no protection tests were carried out with the immune serum absorbed with these fractions, so it is impossible to make a close comparison of the results with those of Enders.

Small doses of the Type I cellular carbohydrate were effective, by intraperitoneal injection, in protecting mice against a fatal

dose of culture administered seven days after the immunizing dose. No data were given as to the degree of immunity established or as to its duration.

The cellular carbohydrate caused an acute inflammatory process in the lung of the rabbit when introduced into the trachea. It also induced specific anaphylactic shock in sensitized guinea pigs. The cellular carbohydrate therefore agreed in its properties and activities somewhat closely with the A substance of Enders and appeared to be a substance apart from the soluble specific substance of Heidelberger and Avery, and from the C substance of Tillett and Francis.

COMPARISON OF VARIOUS CELLULAR CARBOHYDRATES

For the purpose of comparing the properties of the various cellular carbohydrates derived from Pneumococcus, Wadsworth, Crowe, and Smith[1469] studied the absorption spectra of preparations at different stages of purification. The preparations studied were those which had been used in chemical and immunological studies previously reported from their laboratory and, in addition, a specifically reacting substance from an atypical strain of Pneumococcus, originally derived from a virulent Type I standard culture, together with a Type I soluble specific substance from the virulent culture. The absorption curves of solutions of the soluble specific substance of a virulent and of an attenuated Type I strain showed no significant differences. The curve of one preparation of a Type I cellular carbohydrate approximated that for the soluble specific substance, but that for another preparation of a Type I cellular carbohydrate approached the curve of the atypical strain of Pneumococcus.

The authors pointed out that there was considerable evidence in the literature that a large number of carbohydrates show only continuous absorption in the ultra-violet region of the spectrum but, when contaminated even slightly, yield marked absorption bands. Their work led to a contrary conclusion, since it was found that

the selective absorption in the ultra-violet region was character-
istic of the pure substance. In conclusion, Wadsworth, Crowe, and
Smith stated, however, that the curves suggested that since their
preparations were not in a pure state, the more striking differences
in the curves might be attributable to substances not concerned in
serological or antigenic activity or in the production of purpura.

TYPE VIII CARBOHYDRATE

Brown[157] has published a description of a preparation of the
Type VIII carbohydrate isolated by the methods employed by
Wadsworth and Brown. She found that the soluble specific sub-
stance was best removed from the broth concentrates of Pneumo-
coccus by precipitation as the barium or calcium salt and by re-
peated alcoholic precipitation. On analysis one preparation gave a
nitrogen content of 0.19 per cent, phosphorus 0.06 per cent, ash
0.70 per cent, with 3.90 per cent moisture. The specific rotation
was about $+126°$, and while the substance before hydrolysis failed
to reduce Fehling's solution, after boiling for four hours with 10
per cent sulfuric acid, it yielded 69.5 per cent of reducing sugars
calculated as dextrose.

This VIII carbohydrate in a dilution of 1 to 4,000,000 gave a
precipitate with Type VIII antiserum, and in a dilution of 1 to
2,000,000 with Type III serum. When Type VIII serum was ab-
sorbed with Type III soluble specific substance, it still precipitated
with Type VIII carbohydrate in the same dilution as before ab-
sorption; but, when the same serum was absorbed with the homolo-
gous carbohydrate, it failed to precipitate with either. Also, Type
III SSS removed from Type III antiserum the precipitins for both
Types III and VIII polysaccharides, but Type VIII carbohy-
drate removed only the homologous precipitins.

This new carbohydrate produced purpura in mice but its action
was partly neutralized by Types III and VIII antipneumococcic
rabbit serum and was intensified by two similar antiserums from

the horse. The Type VIII polysaccharide also induced fatal anaphylactic shock in guinea pigs passively sensitized with the homologous antiserum from the rabbit but not with a similar serum obtained from the horse.

Among the many methods applied to the purification of pneumococcal carbohydrate, ultrafiltration was employed by Brown.[158] By passing broth cultures of Type VIII Pneumococcus through a Sharples centrifuge and then filtering the clarified effluent through parlodion filters, or by passing broth cultures through a single nitrocellulose-coated alumina thimble, the author effected the elimination of inert material with a consequent enhancement of serological activity.

ISOLATION OF AN UNIDENTIFIED CONSTITUENT OF THE PNEUMOCOCCAL CELL

In the interval elapsing between the first and third papers of Wadsworth and Brown, Felton[413] reported the isolation of what he claimed to be yet another constituent of the pneumococcal cell. This derivative was described as a non-carbohydrate and probably a non-protein substance, possessing the ability to immunize mice against pneumococcal infection. Supplementing his preliminary announcement, Felton[417] in October, 1934, published information which, to a considerable extent, clarified the doubt concerning the true nature of his antigenic "non-polysaccharide and probably non-protein derivative" of Pneumococcus. In the earlier paper, Felton reported that this crystalline substance gave a negative biuret test, a negative Molisch reaction, and also failed to cause precipitation in antipneumococcic horse serum. Further study, however, showed "that the crystalline material lacked immunizing properties, and that in all likelihood the crystals were leucine. In addition, although the substance gave negative Molisch reaction, it was found that the difference in the reduction of copper before and after hydrolysis corresponded to from 0.25 to 0.5 per cent glu-

cose. In other words there is present in this fraction a complex carbohydrate."*

Felton[417] amplified the study of the acid-soluble and acid-insoluble fractions of Pneumococcus on which he had already published a preliminary note.[416] The material was prepared by dehydrating broth-grown pneumococci with acetone, followed by dessication *in vacuo* over calcium chloride. Prepared by this method the organisms retained their antigenicity at least through the period of the study. Watery suspensions of the dried pneumococci were treated first with enough sodium hydroxide to make the concentration of the alkali one-tenth normal, and then after the suspensions were allowed to stand at room temperature for one-half hour, an equal volume of various mineral and organic acids was added. In this way it was possible, as Heidelberger and Avery, and Wadsworth and Brown had found, to separate the pneumococcal material into an acid-soluble and an acid-insoluble fraction. The former possessed most of the immunizing activity of the cell, and the immunity produced by injecting this fraction into white mice proved to be largely type-specific. The latter fraction, probably containing some intact cells, also possessed a small amount of the immunizing substance and evoked in mice a heterologous immunity. Precipitation of the acid-soluble fraction with ethyl alcohol or acetone yielded at least 90 per cent of the immunizing substance.

Felton's experiments, therefore, confirmed the work of Schiemann and Caspar,[1228] Saito and Ulrich,[1214] Wadsworth and Brown,[1466] and Zozaya and Clark[1590] in that active immunity can be produced in white mice by a fraction smaller than the intact pneumococcal cell. The precipitating action of the acid-insoluble

* In another part of the same paper, Felton stated that in his experience the Molisch test failed in the presence of other organic substances to indicate the presence of polysaccharide which on hydrolysis gave a glucose content of from 0.2 to 0.5 per cent, and emphasized the fact that the biuret test for protein is notably insensitive, giving a positive test with concentrations of the majority of proteins in a dilution no higher than 1 to 10,000. When one considers the extraordinary immunological activity of bacterial polysaccharides and proteins, it need scarcely be said that less reliance should be placed on these or similar tests as criteria for determining the precise chemical nature of these antigens.

fraction was also similar to that of the C Fraction of Tillett and Francis, but Felton ventured no closer comparison of the immunizing principle in the acid-soluble fraction with the soluble specific substance of Heidelberger and Avery.

Looking back on the descriptions of the properties of the original soluble specific substance of Heidelberger and Avery, this array of diverse pneumococcal carbohydrates was confusing. One wondered which, if any. of these several substances obtained from Pneumococcus actually represented the specific polysaccharide as it existed preformed in the bacterial cell, or which one most closely approached the native substance in its chemical and antigenic features. There was the possibility, of course, that Pneumococcus might contain more than one constituent of this general type, with differences in individual composition which would account for their special immunological properties.

CAUSE OF DIFFERENCES IN CARBOHYDRATES ISOLATED FROM PNEUMOCOCCUS

In the brief communication by Pappenheimer and Enders[1049] published in October, 1933, there was a definite clue to the cause of chemical and antigenic differences in some of the various carbohydrates isolated from Pneumococcus. Enders had previously observed that the immunological activity of the A substance was rapidly destroyed by heating on the alkaline side of neutrality, and so Pappenheimer and Enders surmised that in the method of Heidelberger and Avery for the preparation of soluble specific substance the A substance was destroyed at the stage where it was precipitated with barium hydroxide.* Pappenheimer and Enders, in preparing the specific polysaccharide, using the simplified method of Heidelberger and Kendall,[620] accordingly took the precaution of maintaining an acid reaction throughout the process. In this way there was obtained from Type I Pneumococcus an extremely hy-

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* Dudley and Smith[340] also noted that heating with alkali destroyed the precipitating activity of a preparation of pneumococcal polysaccharide made by their method.

groscopic, white, amorphous powder, soluble in the range pH 1 to 9, and containing no sulfur or phosphorus. Its elementary analysis, amino nitrogen content, and specific rotation were practically identical with the soluble specific substance as prepared by Heidelberger, Goebel, and Avery.

The soundness of the reasoning was shown by the outcome of precipitin tests with this new preparation of the A substance and SSS. Both, in a dilution of 1 to 4,000,000, precipitated Type I immune serum. When the serum was absorbed with soluble specific substance, whereas SSS no longer gave a precipitate, the A substance produced a precipitate with the SSS-absorbed serum even when added in a concentration of 1 to 4,000,000. On the other hand, the A substance completely removed the precipitin for both itself and the soluble specific substance. The authors had, by obviating the injurious effect of alkali on the carbohydrate, succeeded in largely preserving the chemical and antigenic integrity of the specific polysaccharide of Type I Pneumococcus, and had supplied an explanation for one of the basic causes of the varied immunological behavior of the different carbohydrate preparations previously reported.

RELATION OF THE ACETYL GROUP TO THE IMMUNOLOGICAL ACTIVITY OF PNEUMOCOCCUS

The diversity of the several polysaccharides had also naturally perplexed Avery and Goebel who, quite independently of Pappenheimer and Enders and while their work was in progress,* set about the acquisition of a fuller knowledge of the nature of the relationship existing between the specifically reacting derivatives studied by other investigators and the type-specific polysaccharide formerly described by Avery and his colleagues. In a communication published in 1933 Avery and Goebel[46] presented evidence that they had isolated the soluble specific substance in a chemical form

* The paper by Pappenheimer and Enders appeared in October, 1933, and that by Avery and Goebel in December of the same year.

more closely approximating that in which it probably exists as a natural constituent of the cell capsule. They identified the type-specific carbohydrate present in the intact bacterial cells and in filtrates of autolyzed broth cultures as an acetyl polysaccharide. According to Avery and Goebel this naturally occurring acetyl polysaccharide differs chemically from the specific carbohydrate as originally isolated principally in respect to the presence of acetyl groups, which endow the native substance with additional specific properties not possessed by the polysaccharide after the removal of these labile groups by alkaline hydrolysis. Avery and Goebel claimed that, owing to the marked instability of the acetyl groups and the ease with which they are removed by treatment with alkali, the soluble specific substance as originally isolated, although still retaining the dominant type-specificity of the native substance, had, through the loss of its acetyl groups, suffered a corresponding loss of certain specific properties possessed only by the acetyl polysaccharide itself. Avery and Goebel further stated that the specific differences between the properties of the cell fractions studied by other investigators and those of the soluble specific substance as originally defined, appeared, as a result of their experiments, to be due to the presence or absence of acetyl groups in the polysaccharide molecule. They wrote: "Indeed, so distinctive are the immunological reactions of the acetyl polysaccharide and those of the deacetylated derivative, that it is now possible to clarify many of the apparently conflicting views still current concerning the nature and properties of the specific carbohydrate of Pneumococcus Type I."

By modified methods, in which treatment with alkali was purposely avoided, the soluble specific substance of Pneumococcus Type I was isolated by Avery and Goebel in the form of an ash-free, acetyl polysaccharide possessing marked acidic properties. It was readily soluble in water, and gave solutions of high viscosity which showed a specific optical rotation of about $+270°$. The naturally acetylated Type I polysaccharide was found to contain

4.85 per cent of nitrogen, approximately one-half of which was liberated in the amino form when the substance was treated with nitrous acid in the cold. It did not reduce Fehling's solution until after hydrolysis with dilute mineral acids. At the same time that reducing sugars appeared in the solution, the serological specificity of the acetyl polysaccharide was destroyed. In this respect its behavior was identical with that of the deacetylated polysaccharide.

The acetylated carbohydrate evidently contained uronic acids. It was soluble in water and in 80 per cent acetic acid. Aqueous solutions were precipitated by phosphotungstic acid, silver nitrate, and neutral and basic lead acetate, and were incompletely precipitated by barium hydroxide. Unlike the deacetylated product, the acetyl polysaccharide was precipitated by tannic acid but not by uranyl nitrate. It gave no color reaction with iodine-potassium iodide solution. It did not immediately decolorize weak solutions of potassium permanganate, and it gave negative reactions to the biuret, ninhydrin, sulfosalicylic, and picric acid tests. No traces of phosphorus or sulfur were detectable in the most highly purified preparations of the specific acetyl polysaccharide.

The table on page 281, copied from the article by Avery and Goebel, shows the results of the analyses of the acetyl polysaccharide of Pneumococcus Type I.

The analytical data presented in the table show that the deacetylated product (Preparation 2 A), obtained by alkaline hydrolysis of the acetyl polysaccharide (Preparation 2), contained no acetyl groups and was in all respects chemically identical with the polysaccharide that had hitherto been known as the soluble specific substance. This result agrees with that of Heidelberger and Kendall[617] who previously had found that the Type I polysaccharide (deacetylated) contained no acetyl groups.

The results of the analysis corresponded closely with the calculated composition of the acetylated polysaccharide, and this substance, therefore, approached in its chemical make-up that of the

ANALYSES OF THE ACETYL POLYSACCHARIDE OF PNEUMOCOCCUS TYPE I

Preparation No.	Source	Acid equivalent	Specific rotation	Ash	C	H	Total nitrogen	Amino nitrogen	Acetyl	Phosphorus	Reducing sugars after hydrolysis	Highest dilution of polysaccharide reacting with antipneumococcus serum
				Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	
1	Bacterial cells	—	+270°	0.0	—	—	4.89	2.30	5.9	0.0	—	1:5,000,000*
2	" "	—	+265°	0.0	—	—	4.86	2.21	6.9	—	—	1:5,000,000*
3	Autolyzed broth cultures	576	+277°	0.0	42.55	6.58	4.85	2.22	6.0	0.0	32.0	1:5,000,000*
2 A, deacetylated	†	535	+297°	0.0	‡	‡	5.05	2.50	0.0	—	27.6	1:5,000,000§

* Type I antipneumococcus serum previously absorbed with Preparation 2 A (deacetylated).

† This sample of deacetylated polysaccharide was obtained by alkaline hydrolysis of Preparation 2. This material is identical with the carbohydrate formerly known as the soluble specific substance of Type I Pneumococcus.

‡ An analysis of carbon and hydrogen was made on a sample of deacetylated carbohydrate which had been reprecipitated five times at its isoelectric point. The material contained no ash, and had a carbon content of 40.33 per cent and a hydrogen content of 6.23 per cent.

§ Unabsorbed Type I antipneumococcus serum.

soluble specific substance as it probably exists in the pneumococcal cell. This hypothesis is further strengthened by the antigenic behavior of the acetylated polysaccharide when compared with that of the intact pneumococcus and with that of the soluble specific substance as it is released from the cell during natural autolysis. The experimental evidence presented by Avery and Goebel goes a long way toward reconciling the conflicting differences in some of the various carbohydrate derivatives isolated by other workers from the pneumococcal cell and its products.

The same authors found that both the acetyl polysaccharide and the deacetylated polysaccharide were precipitated by homologous immune serum in the highest dilution tested, representing a final concentration of one part in three million. When, however, the se-

rum was absorbed with the deacetylated polysaccharide, after removal of all precipitins for this form of the specific carbohydrate, the serum still reacted with the acetyl polysaccharide in equally high dilution. On the other hand, after absorption with the acetyl polysaccharide, the serum was completely exhausted of all precipitins for both forms of the carbohydrate, as shown by the absence of reaction when tested with each substance in dilutions ranging from 1 to 20,000 to 1 to 3,000,000. The deacetylated polysaccharide, therefore, selectively removed from the serum only the precipitins for itself, whereas the acetyl polysaccharide completely removed all the precipitating antibodies for both forms of the specific substance.

Avery and Goebel discussed these results by saying:

The specific precipitation of the acetyl polysaccharide in serum previously absorbed with the deacetylated carbohydrate, and the readiness with which the former substance is converted into the latter by heat, are similar to the relationships observed by Enders, and by Wadsworth and Brown, between the substances isolated by them and the soluble specific substance which they prepared according to methods previously described in this laboratory. Since the specific substance thus prepared is now known to be the deacetylated polysaccharide, it seems not improbable that the differences they observed, like those noted in Table II, represent the reactions not of two different carbohydrates but of a single substance in two chemically different forms; namely, the naturally acetylated and the artificially deacetylated polysaccharide.

Avery and Goebel also found that after absorbing Type I antipneumococcic serum separately with acetyl and deacetylated Type I polysaccharides, the serum absorbed with the former carbohydrate no longer agglutinated Type I pneumococci, while immune serum treated with the latter substance still contained specific agglutinins. The authors next tested the ability of the acetyl and the deacetylated polysaccharides to absorb the protective antibodies from specific immune serum and discovered that while the deacetylated carbohydrate reduced the titer of protective antibodies, it failed to remove them all, since the serum, absorbed by this sub-

stance, according to the conditions of the test, in dilutions of 1 to 10 to 1 to 100 still protected mice against virulent pneumococcal infection. On the contrary, the acetyl polysaccharide, under the experimental conditions employed, exhausted the serum of protective antibodies.

Further evidence of the specific antigenicity of the acetyl polysaccharide as compared to the incomplete antigenic action of its deacetylated derivative was furnished by Avery and Goebel's experiments on the immunizing action of the two carbohydrates on mice. Three injections of 0.5 cubic centimeters each of a 1 to 2,000,000 solution of the acetyl polysaccharide protected all the mice tested six days later against 10^{-5} cubic centimeters of a culture of Type I Pneumococcus, of which 10^{-8} cubic centimeters killed the control mice. A similar series of injections of the same amounts of the deacetylated polysaccharide, however, failed to afford any protection to the mice tested.

The outcome of the experiments just cited would explain the previous consistent failure of Avery and his associates to induce active immunity in mice and rabbits with their former preparations of the soluble specific substance, which were then used only in the deacetylated form. "This difference in antigenic action, like that already noted in the serological behavior of the two forms of the polysaccharide, is referable to known differences in chemical constitution."*

A careful examination of the chemical and immunological data presented in this communication would seem to justify Avery and Goebel's conclusion:

An analysis of the specific reactions of the acetyl polysaccharide discloses a previously unsuspected similarity between this form of the specific carbohydrate and the antigenically active fractions described by other investigators. From the chemical and immunological properties of the acetyl polysaccharide it seems highly probable that this substance in the purified state accounts for the antigenic action of the carbohydrate

* Avery and Goebel.

of Schiemann and Caspar and of Wadsworth and Brown. As in the case of these substances, the acetyl polysaccharide is antigenically effective in mice only when administered in extremely minute quantities. Although an extensive study of the purpura-producing action of the acetyl polysaccharide has not been made, in several instances purpura has been noted in mice injected with amounts of this substance ranging from 0.4 to 4.0 mg. . . . That the antigenic action of the water-soluble fraction of Perlzweig and his co-workers may have been due to the presence of traces of unhydrolyzed acetyl polysaccharide seems not unlikely from the readiness with which it lost its immunizing capacity when heated in alkaline solution.

The correspondence of the acetyl polysaccharide to the A substance of Enders and to the cellular carbohydrate of Wadsworth and Brown has already been mentioned. The exact relation of the acetyl polysaccharide to the "non-carbohydrate and probably non-protein" derivative of Pneumococcus described by Felton still remains to be determined.

Avery and Goebel had found, under the conditions of their experiment, that the acetylated polysaccharide failed to induce any immune response in rabbits. The serum of the treated animals contained no demonstrable antibodies, and the animals were not protected against subsequent infection with organisms of the homologous type. It was shown, furthermore, that the acetyl polysaccharide persisted in the circulation of the treated rabbits for considerable periods of time, was slowly excreted by the kidney, and appeared in the urine in its naturally acetylated form. This observation, particularly when compared to the immunizing action of the same substance in mice, has an important bearing on the definition of antigenicity.

In 1934, Goebel, Babers, and Avery[523] sought a better understanding of the immunological significance of the acetyl group in these complex pneumococcal polysaccharides. For this purpose the authors synthesized the p-aminophenol β-glucoside of glucose and its monoacetyl ester and then combined these two glucosides with horse serum globulin by means of the diazo reaction. These syn-

thetic carbohydrate-azoproteins were employed as antigens in the production of immune rabbit serum. From the experiments, involving homologous precipitation and specific inhibition of precipitation, confirmation was obtained of the view previously expressed by Avery and Goebel that the immunological specificity of carbohydrates is determined by their stereochemical configuration, and their data lent support to the further assumption that the introduction of a simple chemical group, such as the acetyl radical, endows a carbohydrate with a new and distinct specificity which is determined by the chemical nature of the group thus introduced. The differences exhibited by these two purely synthetic carbohydrate azoproteins accurately paralleled the differences in the serological specificity exhibited by the acetylated and deacetylated polysaccharides of Type I Pneumococcus.

Additional information concerning the A substance of Enders was presented in the 1934 communication of Enders and Wu,[362] who prepared the A substance according to the procedure given by Pappenheimer and Enders and the soluble specific substance by the method of Heidelberger, Goebel, and Avery. The immune serum was obtained by the repeated intravenous injection of rabbits with Type I pneumococci in 0.3 per cent formalinized saline solution, and also of suspensions of pneumococci killed by heating at 56° and 60°. The immunological properties of the two polysaccharides were then tested by the bactericidal method of Ward and by mouse-protection tests in which the mice had been treated with antipneumococcic rabbit serum and also by active immunization with the A substance.

After the completion of the study, Enders and Wu announced that the A substance possessed greater anti-opsonic action than either the deacetylated carbohydrate obtained by boiling in alkali or the SSS of Type I Pneumococcus prepared according to the method of Heidelberger, Goebel, and Avery. The A substance practically eliminated the opsonic titer of normal human serum— an effect not observed with equivalent amounts of the deacetylated

material or the soluble specific substance—while in immune serum
the A substance brought about a quantitatively greater reduction
in opsonic activity than its derivatives, although the authors were
not able to demonstrate complete inhibition of phagocytic action
by the method of absorption of antibody. The A substance by ab-
sorption lowered the mouse-protective titer of Type I antipneumo-
coccic rabbit serum to a greater degree than did a similar treat-
ment with the deacetylated carbohydrate. Analogous to the acetyl
polysaccharide of Goebel and Avery, the A substance, adminis-
tered in very small quantities, protected mice against an otherwise
fatal dose of Type I Pneumococcus, although doses larger than
0.005 milligrams failed to establish protection in the animals. This
particular antigenic action of the A substance was impaired by
boiling in 0.02N sodium hydroxide, and was destroyed by similar
treatment with 0.1N sodium hydroxide.

Enders and Wu also determined that after the injection of the
A substance into mice active immunity arose within three days fol-
lowing the injection, reached its height in from six to twenty-five
days thereafter, and became retrogressive by the forty-ninth day
following vaccination. As might be expected, the injection of the A
carbohydrate into immunized mice immediately before giving an
infective inoculation abolished the active immunity, while the serum
of mice actively immunized with the A substance conferred passive
immunity on normal mice. The authors suggested that, since the
evidence which had accrued in the course of their study indicated
that the A carbohydrate obtained from Type I Pneumococcus and
the acetyl polysaccharide of Avery and Goebel represented the
same chemical substance, the designation A carbohydrate or A
substance be relinquished in favor of the more accurately descrip-
tive term, acetyl polysaccharide.

Although Enders and Wu did not include the cellular carbohy-
drate of Wadsworth and Brown in their comparison, taking into
consideration their observations together with those of Avery and
Goebel, there is good reason to believe that some of the prepara-

tions of cellular carbohydrates, though presumably in impure form, are practically identical with the A substance and the acetyl polysaccharide. There is, nevertheless, one point of difference for which as yet no explanation has been forthcoming, and that is the presence of sulfur and phosphorus reported by Wadsworth and Brown in their preparations. Heidelberger never found sulfur or phosphorus in the specific polysaccharides of Type I, II, and III pneumococci and since Avery and Goebel have stated that their highly purified preparation contained neither of these elements, it becomes difficult to reconcile these differences.

The work of Enders and Wu, taken with that of Avery and Goebel, would seem to demonstrate that the A substance and the soluble specific substance approached very closely in antigenic function the hypothetical specific polysaccharide as it exists in the intact pneumococcal cell. There developed in the latter's study one point, however, which raises a doubt as to the exact common identity of the isolated specific carbohydrate and the native capsular polysaccharide of Pneumococcus. The fact that the former, by absorption, failed to remove all the protective antibodies from the homologous immune serum might be taken to mean that in its isolation it had suffered the loss of some completing molecular group, or possibly that its full antigenic power is exerted only when it is in combination with some other constituent of the pneumococcal cell.

Another striking difference between the acetyl and the deacetylated polysaccharides of Pneumococcus was manifested in the behavior of these two derivatives toward the blood-group specific substance A. Witebsky, Neter, and Sobotka[1526] in 1935 announced that a relationship between the soluble specific substance of pneumococci and the blood-group substance A of man could be demonstrated by the inhibition of sheep-cell hemolysis by a group-specific A-antiserum, although the various types exhibited certain quantitative differences. When, however, the deacetylated carbohydrate was used, it failed to react with the group-specific A-antiserum,

while the acetyl polysaccharide, under the conditions of the test, exerted an inhibitory influence on sheep-cell hemolysis by the A-antiserum up to a dilution of 1 to 1,000,000 of a one per cent solution. Furthermore, the authors could demonstrate the activity of the acetyl polysaccharide by complement fixation and by inhibition of group-specific iso-agglutination.

Witebsky, Neter, and Sobotka reported another interesting observation on the properties of the acetyl polysaccharide. When this substance was treated with the feces filtrate previously described by Schiff and Akune,[1233] by Schiff and Weiler,[1234] later by Witebsky and Satoh,[1527] and still more recently by Sievers,[1285] it lost much of its inhibitory action toward the group-specific A-antiserum, and also its ability to inhibit the iso-agglutination of Group A blood cells. Moreover, the acetyl polysaccharide of Type I Pneumococcus, after having lost its reactivity toward the group-specific A-antiserum following treatment with feces filtrate, still reacted with Type I antipneumococcic serum that had previously been absorbed with deacetylated Type I polysaccharide.

Witebsky, Neter, and Sobotka,[1526] in the introduction to their communication, mentioned the correlation between the Forssman antigen and the blood-group specific substance of human blood-group A. This fact, considered along with the relation existing between pneumococci and the blood-group specific substances A and B as reported by Bailey and Shorb,[66] and the known carbohydrate nature of the blood-group substance A as demonstrated by Landsteiner,[780] Landsteiner and Levene,[781] and by Brahn, Schiff and Weinmann,[146] and then the isolation by Freudenberg and Eichel[482] from the urine of men belonging to group A of a carbohydrate closely resembling in its chemical structure the type-specific polysaccharide of Pneumococcus of Avery, Heidelberger, and Goebel, reveals a field of investigation which yet remains to be explored.

RECENT METHODS OF PREPARING CAPSULAR POLYSACCHARIDE

The effect of alkali in impairing or destroying the antigenicity of the specific polysaccharides of Pneumococcus was tested by Fel-

ton (1934),[416] who reported that a Type I immunizing antigen, when heated at 100° in 0.1N sodium hydroxide lost 50 per cent of its antigenic strength after one hour of heating, and 94 per cent in two hours. With a Type II antigen in 0.5N sodium hydroxide, the immunizing activity remained the same as that of the control after four hours' heating. The same sample of Type I antigen heated in 0.1N acetic acid for two hours lost 50 per cent and in 0.1N hydrochloric acid 87 per cent of its immunizing power. Its precipitating action, however, remained the same. Of the samples of SSS prepared by the technique of Avery and Goebel and heated under the same condition, Type I in one test showed destruction of antigenicity and, in a second, no loss; with Type II no loss was observed. In a miscellaneous group of pneumococcal fractions with immunizing activity in dilutions of 1 to 50,000,000 to 1 to 2,000,000, and with variation of hydrolyzable sugar from 12 to 0.25 per cent, absorption end-point from 8 to 0.1 per cent, and precipitin titer from 1 to 5,000,000 to 1 to 5,000, Felton found that there was no definite correlation between acetyl group content and immunizing activity. However, acetyl groups were found in the samples studied ranging from 6.4 per cent in one sample which immunized in a dilution of 1 to 5,000,000, to 1.2 per cent in a sample which immunized in the same dilution. Inasmuch as this information was published merely in abstract form without the presentation of any experimental data, it is impossible to set a proper value upon its significance.

In 1935, Felton, with Kauffmann and Stahl,[430] gave the details of a method that had been employed in their laboratory for five years in the routine preparation of soluble specific substance of pneumococci. It had been planned to eliminate the time-consuming initial evaporation of the broth culture and to minimize any alteration of the chemical and antigenic structure of the carbohydrates. Calcium phosphate was employed as a selective adsorbent for separating the polysaccharide from the culture medium and its constituents.

Felton and his co-workers stated that the product from the first

had shown characters differing from those of the soluble specific substance of Heidelberger and Avery. The new preparation precipitated more protein from a given immune serum; it consistently removed all the protective antibody from an homologous serum; and it produced immunity in white mice. The authors were not inclined to grant the validity of Avery and Goebel's explanation that this immunizing property depended upon the presence of the acetyl group in the polysaccharide. They had, it seems, in a comparative test succeeded in actively immunizing white mice not only with their preparations but also with a sample of soluble specific substance prepared by Heidelberger by his original method. Some statement as to the presence or absence of the acetyl group in their and Heidelberger's own preparation would have been helpful in arriving at a just appraisal of their contention.

In the latest paper to come from Felton's laboratory (1936), its authors (Felton and Prescott)[431] present evidence which, in their opinion, challenges the validity of the theory that the specific antigenic properties of pneumococcal polysaccharides are due to the presence of the acetyl group in the molecule. Because of the seeming heterodoxy of the claim, the summary and conclusions are here repeated practically verbatim:

A method has been indicated by which the linkage between the units in the polysaccharide of Type I pneumococcus are altered with concurrent changes in biological activity. This alteration is shown by both the high titer in bisulfite and iodine reactions in the original material and the absence in samples B* and C* of reducing sugars on hydrolysis after destruction of the aldehyde groups in hot NaOH solution.

* Sample A was a purified preparation consisting of a mixture of seven samples of Type I polysaccharide prepared by different methods both from the supernatant broth (of pneumococcal cultures) and from the bacterial cell (Pneumococcus).

Sample B was the original A dissolved at pH 7 in a concentration of 1 mg. per cc., and then made alkaline to N/10 concentration with NaOH and heated in an Arnold sterilizer at 100°C. for 30 minutes. To the alkaline solution after cooling were added 2 volumes of a 1:1 alcohol-ether mixture. The white precipitate which formed was washed thoroughly with alcohol, alcohol-ether, and dried.

Sample C was made from the foregoing fraction (B) by treating a neutral aqueous solution, containing 1 mg. per cc., with one-tenth of the volume of concentrated NH₄OH at 4°C. for 18 hours. The NH₄OH solution was precipitated

From this altered SSS, four samples were prepared by various alkaline treatments and tested both chemically and biologically in comparison with the original material. Each of the samples may be considered separately. The original material (A) gives tests for aldehyde groups, is high in "acetyl" content, and is optically as well as immunologically active. In B, a portion of A treated for 30 minutes in N/10 NaOH at 100°C., the optical activity is lost, the aldehyde groups are removed, "acetyl" content and all immunological activities are greatly reduced. In C, sample B treated with NH_4OH, there is a reduction of acid from vacuum distillation, no indication of sugar on acid hydrolysis, but an increase of the iodine reaction. Optical rotation is changed from zero to +190°, or higher than the control. Immunologically also this sample is more active than the original material (A). Sample D is the original material (A) treated with NH_4OH. There is a decrease of 90% in acid on vacuum distillation, a slight decrease in glucose number, a higher optical rotation than the original material, as well as a high titer in all immunological tests. It is significant that with a 90% decrease in "acetyl" content the immunizing activity is higher than the original. There is also a significant increase in its combining equivalent with antibody. Sample E is the NH_4OH treated control (D) further treated with hot NaOH as in sample B. The NH_4OH treatment caused a rearrangement of the molecule or stabilization, for on acid hydrolysis glucose number was 10.25 as compared to zero with sample B. Optical activity was reduced but not entirely lost. Immunological tests, although decreased, were not as low as in sample B, with the exception of the combining equivalent and that was the lowest of all samples, 0.9 unit. The acid from vacuum distillation was higher than in sample D, indicating a splitting off of the terminal carboxyl radicals.

At this stage of our investigation certain inferences may be made: (1) An antigen has been prepared from a Type I polysaccharide which in our opinion is non-protein in nature for the following reasons: (a) treatment with hot NaOH (N/10) and then NH_4OH results in a product more highly antigenic than the original preparation, and (b) all well-recognized protein tests are negative including a test for sulfur with a large sample of material. (2) This antigen may be considered

by dilution with 2 volumes of 1:1 alcohol-ether, washed repeatedly with alcohol, alcohol-ether and dried.

Sample D was made from the original material (A) by treatment with NH_4OH as in C.

Sample E was prepared from D by the same procedure as B, in other words heating at 100°C. in the Arnold sterilizer in N/10 NaOH for 30 minutes.

non-polysaccharide since both the Molisch test and the test for reducing sugars after acid hydrolysis are negative. (3) The antigens so prepared from Type I SSS produce active immunity in mice against both Type I and Type II pneumococci. (4) The restoration of the biological properties destroyed with hot NaOH by treatment with NH_4OH shows that the "acetyl" content is of no significance in determining the biological activity of the preparation studied, but conversely indicates that this property is determined by a definite molecular configuration which is readily altered by strong alkalis.

Believing that the procedures hitherto employed in the preparation of pneumococcal polysaccharides might have disrupted the molecular configuration of the carbohydrate molecule with a consequent loss of essential radicals, Sevag (1934)[1257] applied gentler measures for their isolation. Adopting the well-known action of liquid air in disintegrating bacterial cells, Sevag first froze the sediment from twelve-hour dextrose-serum broth cultures of a virulent strain of Type I Pneumococcus and then subjected the detritus to prolonged shaking in a mixture of water, chloroform, and amyl alcohol. The protein coagulated by this treatment was removed and the polysaccharide in the supernatant fluid was isolated by precipitation with alcohol. After further purification with chloroform, an alcohol-insoluble fraction was obtained which was claimed to be protein-free, gave a strong Molisch reaction, and contained between 6.60 and 6.72 per cent nitrogen, of which from 1.21 to 1.42 per cent was in the form of amino nitrogen. The specific rotation of the product was $[a]$ D $= + 218.3$* to 219.1* which after acid hydrolysis became $+54.1$ and showed a glucose content of 23.98 per cent. The polysaccharide thus isolated, in a dose of 0.0001 milligrams, protected mice against a thousand fatal doses of virulent Type I Pneumococcus and in high dilutions gave a high precipitation titer with homologous immune rabbit serum.

Sevag entertained some doubts concerning the validity of Avery and Goebel's claim that the capsular polysaccharide of Pneumococcus Type I was an acetylated substance and queried whether

* Typographical error in original figures gives $+21.83$ and 21.91.

the acetyl group might have been introduced into the carbohydrate molecule by the repeated treatment of the substance with acetic acid. Sevag accordingly excluded acetic acid from the method of preparing a special sample and found, contrary to expectations, that the polysaccharide, on analysis, had an acetyl content of 7.7 to 7.97 per cent as against the figure of 2.57 per cent obtained by Avery and Goebel.

It might reasonably be argued, therefore, that Sevag, by a less harsh treatment of pneumococcal cellular material, had succeeded in isolating a polysaccharide that more closely represented the carbohydrate as it naturally exists in the cell than did the preparation of Avery and Goebel. Apparent confirmation of the assumption came subsequently with the experimental results of Heidelberger, Kendall, and Scherp (1936).[627] In searching for a reason for the differences in the immunological behavior of acetylated and deacetylated polysaccharides and from the alkali-treated carbohydrates of Type II and Type III pneumococci, the authors devised a method of preparation in which the use of heat, strong acid, or alkali was avoided. In general the procedure consisted of the concentration of culture filtrate *in vacuo* to a convenient volume; separation of the polysaccharide from salts and protein degradation products by repeated precipitation with alcohol in the presence of sodium acetate and acetic acid; removal of proteins by denaturation with chloroform and butyl instead of amyl alcohol, as used by Sevag; and elimination of any glycogen or starch present by methods depending upon the properties of the individual polysaccharides. The products were isolated as the neutral sodium salts.

The analytical data on the polysaccharides obtained by the revised method present many points of interest when compared with results of analyses of older preparations. In the case of Type I polysaccharide the new product contained 4.62 per cent nitrogen with 2.0 per cent in the amino form, against 5.12 and 2.5 per cent for the old; showed specific rotation of +278 against +305, the acetyl content was 7.1 as compared to 3.4; it had a higher vis-

cosity, and precipitated more antibody nitrogen from homologous antiserum from both horse and rabbit than did the earlier products. When compared with the substance isolated by Sevag, the high nitrogen, low amino nitrogen, and optical rotation indicated to Heidelberger and his associates the presence of a nitrogen-containing component not present in their products, but there was at the time no way of determining whether the component was an impurity or an integral part of the polysaccharide as it exists in the organism.

The study of Heidelberger, Kendall, and Scherp also revealed that, contrary to earlier opinion, the specific polysaccharides are not thermostable. On heating preparations from pneumococci of Types II and III there was an accompanying and marked drop in viscosity without any change in reactivity with homologous antiserum. There was a decrease in precipitating power in the case of Type I polysaccharide owing to a partial removal of acetyl. The authors suggested that unheated preparations have the largest particle size or longest chain, and that heating results in a degradation of the molecule to smaller units.

In the same communication the authors reported the actual isolation of the methyl glucoside of galacturonic methyl ester from the products of hydrolysis of the Type I polysaccharide by methyl alcoholic hydrochloric acid.

From the properties of the new preparations, Heidelberger, Kendall, and Scherp believed that while the products might be artifacts just as were the older ones, they were certainly a step closer to the native substances themselves and designated the substances as specific polysaccharides of Types I, II, and III Pneumococcus with the abbreviations SI, SII, and SIII.*

* For the purpose of obtaining a maximal yield of pneumococci with a high polysaccharide content, O'Meara and Brown[1031] devised a medium consisting of peptone, glucose, sodium chloride and sodium bicarbonate, potassium phosphate, and thioglycollic acid in water. In this medium, Type I Pneumococcus grows rapidly and abundantly. The organisms are found to be rich in capsular polysaccharide, whereas the medium contains a minimal amount of free polysac-

The susceptibility of the capsular polysaccharide to even fairly rigorous chemical treatment has been shown further in the case of the soluble specific substance of Type III Pneumococcus by Hornus and Enders.[656] By avoiding as far as possible the use of strong acids in the isolation of the carbohydrate a preparation was obtained that gave a precipitate with Type III immune serum after the serum had been absorbed by Type III SSS made by the earlier method of Heidelberger and Avery. The sample contained 0.3 per cent nitrogen and in this respect resembled the material described by Heidelberger, Kendall, and Scherp,[626] but it differed in some of its serological properties.

The gap between the polysaccharides as derived by chemical manipulation and the native substance in the pneumococcal cell is being still farther narrowed. By omitting the preliminary autoclaving of pneumococcal cultures, by leaving out alkaline treatment, and employing a method that minimized hydrolysis by acid or alkali, Chow[225] obtained a polysaccharide from Type I Pneumococcus that gave a precipitate with homologous immune rabbit serum previously absorbed with the acetyl polysaccharide. The acetyl polysaccharide failed to react with homologous immune rabbit serum after absorption by the new carbohydrate. The greater absorptive power of the new preparation as compared with that of the acetyl polysaccharide suggests its possession of a group or radical which was lacking in the acetylated derivative, but the fact that antipneumococcic serum absorbed with the new preparation was still agglutinative and specifically protected white mice against an otherwise fatal dose of Type I Pneumococcus may be taken to indicate that the carbohydrate isolated by Chow is not so com-

charide. The details of the preparation of the medium are given in the Appendix.

The use of peptone instead of meat as recommended by O'Meara and Brown, according to an unpublished personal communication of Heidelberger, greatly facilitates the preparation of both the capsular polysaccharide and the C carbohydrate. The C Fraction can be easily isolated from the supernatant fluid of such a broth after removal of the capsular polysaccharide.

plete in its antigenic or chemical constitution as the hypothetical polysaccharide existing preformed in the pneumococcal cell. While Chow's statement may be true that his polysaccharide may be the parent substance from which the acetyl polysaccharide could be obtained by appropriate treatment, there is little doubt that further search must be made in the lineage of pneumococcal carbohydrate for the original progenitor of the many derivatives which have been described.

OTHER PHYSICOCHEMICAL PROPERTIES OF THE CAPSULAR POLY-
SACCHARIDE

Heidelberger and Kendall[618] studied other physicochemical properties of the specific polysaccharides of pneumococci, and determined the viscosity, conductance, and behavior in diffusion of the sodium salts of Type III and other capsular polysaccharides. The high values of the equivalent conductance indicated that the sodium salt of Type III S* is a strong electrolyte characterized by a mobile negative ion of very high valence. Under varying conditions of salt concentration the authors determined the viscosities of I, II, and III S and correlated the findings with the magnitude of the charge on the anion. The experimental data indicated that at least eight or ten carboxyl groups were present in the molecule at regular intervals of every 340 of molecular weight, so the cumulative effect of the negative charges on the Type III S would be very large, which should result in large interionic or Coulomb forces. These forces were found to be strong.

The specific polysaccharide of Type I Pneumococcus showed viscosity abnormalities of a similar character, but of smaller magnitude. Although its acid equivalent was even lower than that of Type III S, Heidelberger and Kendall thought that internal compensation of negative charges by the basic groups present was the cause of the smaller effect. Further data on the specific gum arabic

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* The substitution of the letter S for SSS is consistent with Heidelberger's original usage.

and Type III Pneumococcus polysaccharide showed that the viscosity effects decrease with the relative number of carboxyl groups, or negative charges in the molecule.

SUMMARY

It should by no means be assumed that the last word has been written about pneumococcal polysaccharides. Notwithstanding the fact that the presence in any appreciable percentage of what is considered the acetyl group may exert a marked influence on the antigenic properties of these substances, we should not close our minds to the many possibilities which the subject presents. We cannot as yet be sure that any of the substances isolated from Pneumococcus are truly representative of the actual components of the living cell, even though the immunological action of the acetyl polysaccharide most nearly approaches that of the unbroken Pneumococcus.

We can be sure that many of the methods employed up to the present time have led to products far from pure by any standard we set, and we can be equally certain that the majority of the procedures have caused more or less disturbance of the molecular arrangement of the cells' constituents with a consequent loss of chemical and immunological integrity. Many of the preparations hitherto derived from Pneumococcus by natural means must be looked upon as mixtures of carbohydrate, protein, and other cellular elements, and the majority of the preparations produced by chemical methods cannot be accepted as anything but artifacts containing, to be sure, a nucleus of intrinsic antigenic material but, nevertheless, representing substances which eventually may be discovered to be removed in varying degree from the native antigen.

There are chemical groups other than the acetyl radical in pneumococcal capsular polysaccharides, and until we have a more thorough analysis and more exact identification of all these substances supported by their respective immunological reactions, it would be folly to take any dogmatic stand in this highly complex question.

A careful examination of the specifications which have been given in all these reports would enable one to make a tentative and partial chemical reconstruction of the pneumococcal cell. The basic protoplasm would contain protein—a nucleoprotein—with a more or less constant composition for all pneumococci regardless of serological type. This common component would, in some respects, bear a close resemblance to the protein of other members of the genus, *Streptococcaceae,* and a more general resemblance to that of other more distantly related microorganisms. Another constituent of the protoplasm would be lipids, probably combined with the protein and conceivably loosely linked to the carbohydrate. Then the substance of the cell would also include a polysaccharide —a carbohydrate of the nature of the C Fraction of Tillett and Francis. This phosphorus and nitrogen containing carbohydrate would, like the nucleoprotein, be possessed by every Pneumococcus, whether of full virulence or degraded below the stage of any type identity. There would naturally be inorganic salts, but since little is known about them, their actual composition cannot be defined. In this viable, watery solution of colloids and crystalloids there would be enzymes—proteases, lipases, invertases, and saccharases —active in the metabolic processes of the cell and potentially able to destroy it.

This globular mass of protoplasm would be surrounded by a shielding envelope or capsule, largely carbohydrate in nature. The capsular material would be present when the cell was living in a favorable environment, in greatest amount when the surroundings were ideal, but entirely lacking when the cell had suffered from severe degenerative processes. But with or without it, these particular bacterial cells would still be pneumococci, their identity as such resting upon their somatic nucleoprotein and carbohydrate.

The normal Pneumococcus, however, would possess in its capsule a polysaccharide that would serve to distinguish the organism from many of the other members of the species. These complex carbohydrates would differ in their content of nitrogen, of phos-

phorus, and possibly of other elements, some being well supplied with one or the other of these elements, while others would have none. The carbon, hydrogen, and oxygen would be combined to form varying percentages of carboxyl, acetyl, or other radicals, and the presence and proportion of these groups, together with the varying sugars chemically combined, would serve to identify the special serological type to which the pneumococcus belonged. The capsular polysaccharides, furthermore, would differ in their acidic and basic affinities and in their relation to the plane of polarized light. The proportion of these carbohydrates would fluctuate in the different types, and on hydrolysis the substances would yield unequal amounts and various kinds of cleavage products such as glucosamines, sugar acids, and the simpler sugars. With the common protein and the somatic carbohydrate as a nucleus, it would be feasible, as soon as other specific polysaccharides are isolated, to hypothesize a basic reconstruction of all the thirty-two and possibly other as yet undiscovered serological types of pneumococci.

The capsular polysaccharides would be linked to some of the protein, or even to the lipids, in a combination susceptible to cleavage by the enzymes of the cell, to the disruptive effects of physical forces, to the lytic action of bile, or to the hydrolyzing action of chemical agents. Their chemical structure is frail, and to the chemist they present difficulties in the way of isolating them as they exist in their native state. They call for the utmost care in their separation lest their molecular configuration be distorted or mutilated.

While the somatic constituents are sufficient to insure the existence of the cell under ordinary circumstances, the capsular structure composed of type-specific polysaccharides is essential to the complete functioning of the cell as a pathogenic organism, and it is the latter specific complex that gives to a pneumococcus type-specificity and its individual place in the biological and immunological scheme. The capsular material conditions and is associated with the difference between a state of saprophytism and parasit-

ism, and endows the cell with its highly specialized powers for stimulating the tissues of the animal body to the elaboration of substances antagonistic to the type-specific polysaccharide and to the cell as a whole.

The immunological properties of Pneumococcus and of its chemical constituents and derivatives will be discussed in more detail in later chapters.

CHAPTER IX

SPECIFIC POLYSACCHARIDE-SPLITTING
ENZYMES

*The decomposing action of bacterial enzymes on the soluble spe-
cific substance of pneumococci, with a description of the micro-
organisms, of the physical and biochemical properties of the en-
zymes and their effect on pneumococci in the test tube and in
experimentally infected animals.*

THE discovery of the specific polysaccharides of Pneumococcus
was an incentive to a search for enzymes capable of decom-
posing these complex carbohydrates. During pneumococcal infec-
tions in man the soluble specific substance is present in the blood
and since it is excreted unchanged by the kidneys it was reason-
able to expect that none of the body tissues would be able to at-
tack this substance. No tissue has ever been found to possess the
power. Avery and Dubos[42] were the first to make a systematic hunt
for a ferment possessing the ability to digest the capsular poly-
saccharide of Pneumococcus. The authors tested enzymes from
animal and plant sources known to be active in the hydrolysis of
simpler carbohydrates, but all failed to affect the polysaccharides.
The similarity of these bacterial carbohydrates to hemi-cellulose
led the authors to search among the molds, yeasts, actinomyces,
and bacteria known to decompose substances allied to the cellu-
loses, but here again the quest was fruitless.

FIRST ISOLATION OF POLYSACCHARIDE-SPLITTING ENZYME: SIII
BACILLUS

Avery and Dubos reasoned that localities where large amounts
of organic materials—especially materials belonging to the group
of hemi-celluloses—accumulate and undergo decomposition were
most likely to harbor the desired organism. So they tried leaf mold,

composts of corn-cob, rye straw, sphagnum, oak leaves, farm ma-
nure, and soils rich in organic matter, such as peat soils and soils
heavily manured, and finally came to examine a sample of soil from
a cranberry bog. It seemed a far cry from a pneumococcus grow-
ing in the lung of man and, among its other vital activities, build-
ing up a complex carbohydrate, to a lowly saprophytic bacillus
capable of decomposing the specific capsular polysaccharide
formed by Pneumococcus. Such, nevertheless, was the case.

In 1930, Avery and Dubos announced that a mixed bacterial
suspension made from bog soil was able to split the specific capsu-
lar polysaccharide of Type III Pneumococcus, and from the mot-
ley crowd of bacteria in this peat, they succeeded in isolating a
pleomorphic, motile, spore-bearing bacillus that was responsible
for the breaking down of the Type III polysaccharide.

In their next communication, Avery and Dubos[43] gave fuller de-
tails of the isolation, cultivation, vital characters, and enzymatic
action of this curious bacillus. The authors mentioned the earlier
observation of Toenniessen,[1413] who had found that when *Bacillus
vulgatus* was seeded together with encapsulated Friedländer ba-
cilli, the latter organisms grew deprived of their capsule, and then
went on to describe the isolation of the new organism. The mineral
medium used was based on one previously described by Dubos[329]
for the isolation of cellulose-decomposing bacteria, with the addi-
tion of the capsular polysaccharide of Type III Pneumococcus in
final concentrations varying from 0.001 to 0.2 per cent. The pneu-
mococcal polysaccharide was the only source of organic carbon in
the medium.

This mineral medium was selected because it contained no nu-
trient substances that would act as readily available sources of
energy, so that the bacillus, when deprived of any other food,
would attack the specific pneumococcal polysaccharide—an exam-
ple of the so-called "starvation" phenomenon. By repeated trans-
plantation and dilution, and by heating the inoculum at 70°, Dubos

and Avery finally discovered the spore-bearing bacillus which they designated as the "SIII bacillus."

When grown in the synthetic medium, the organism appears as a minute Gram-negative bacillus, at times smaller than the Pfeiffer bacillus. It contains metachromatic granules; it grows diffusely in peptone solution and in this medium sedimentation of the growth occurs after several days' incubation. When cultivated in peptone solution or in plain broth the organism appears as a fairly large, actively motile bacillus, having peritrichous flagellae, the young cells measuring 2 to 3μ by 0.5μ. Short chains and diploforms are often observed. In broth the bacilli are at first Gram-positive and do not autolyze readily, but in the mineral medium the bacilli rapidly become Gram-negative and undergo almost immediate self-digestion. The spores resist heating for thirty minutes at 75° but are killed by boiling for five minutes. On plain nutrient agar free of dextrose, growth occurs in the form of small, whitish colonies, two millimeters in diameter, circular, slightly raised, umbilicated, with entire edge and fairly smooth surface.

In the media tested, the organism is strictly aerobic and exerts its saccharolytic action on Type III Pneumococcus within the range pH 6.2 to 7.8 at room temperature and at 37.5°. The isolated enzyme, however, is equally active under both anaerobic and aerobic conditions, and it is likely that this soluble principle belongs to the group of hydrolytic enzymes.

Dubos and Avery then found that the enzymatic action of the culture could be enhanced by frequent transplantation, and that the enzyme was present in filtered autolysates of the cultures. The action of the enzyme was limited, of all the substances tested, to the Type III polysaccharide, since it failed to decompose the carbohydrates of Types I, II, and VIII* Pneumococcus, and those of Types A, B, and C of Friedländer's bacillus, of *Hemophilus influenzae* Type a, and of gum arabic which, it may be recalled, gives a

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* Personal communication from Dubos.

precipitin reaction with Type III antiserum. The activity of the enzyme was destroyed by heating for ten minutes at 60° to 65°. Neither normal beef nor rabbit serum had any inhibiting effect on its activity.

As a means for measuring the enzymatic strength, Dubos and Avery determined the minimal amount of a given enzyme preparation which would decompose one cubic centimeter of a 0.001 per cent solution of specific Type III capsular polysaccharide in eighteen hours at 37.5°, the decomposition of the carbohydrate being demonstrated by the disappearance of precipitating action in the presence of Type III antipneumococcic serum. As the authors stated, "The specific decomposition of the capsular polysaccharide of Type III Pneumococcus, by the organism as well as by the enzymes it produces, illustrates once more the specificity of the types of Pneumococcus, and confirms the fact that the capsular polysaccharides, and not some impurities carried along with them, are responsible for type-specificity."

EFFECT OF THE ENZYME ON THE CELL CAPSULE

Inasmuch as the capsular substance in its native state forms a morphological structure which conditions the antigenic and serological reactions of the pneumococcal cell as a whole as well as its power to invade and multiply in the animal body, it became of special interest to ascertain what effect this specific enzyme would have on the encapsulated cells growing *in vitro* and *in vivo*. To this end, therefore, Avery and Dubos performed a series of experiments the results of which revealed the fact that the enzyme by itself is neither bacteriostatic, bactericidal, nor bacteriolytic, and that, without impairing the viability of the cocci or without inhibiting the ability of the cells to synthesize polysaccharide, the enzyme, by decomposing the specific carbohydrate, merely deprives the bacteria of their capsules. It was evident that the action of the enzyme did not destroy the function of elaborating the capsular substance, since organisms decapsulated by enzyme again formed
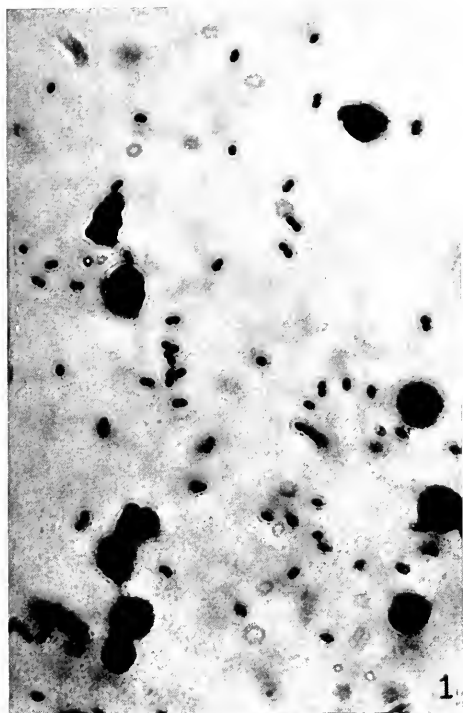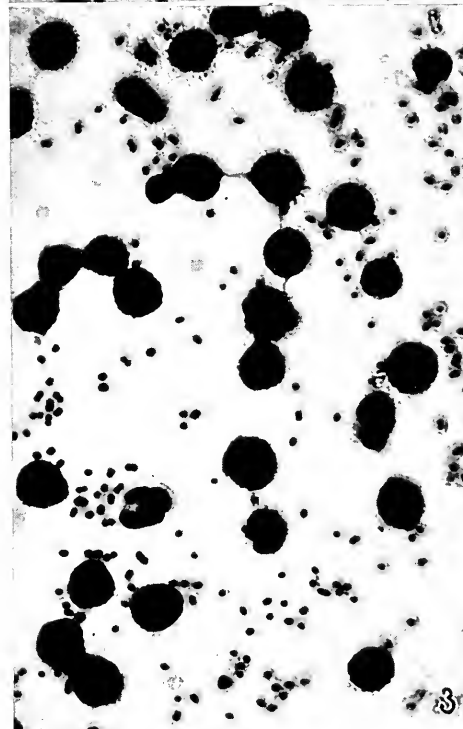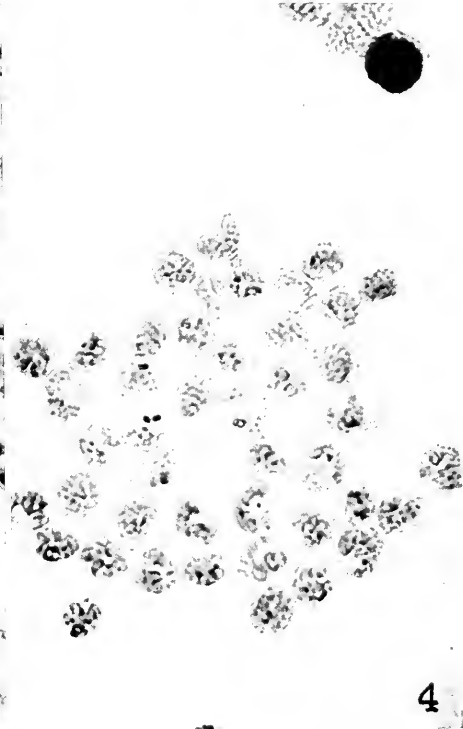
ACTION OF ENZYME AGAINST TYPE III PNEUMOCOCCUS

specific polysaccharide and regained their capsule when transferred to a medium free of the enzyme.

The action of the enzyme against Type III Pneumococcus is shown in the accompanying photomicrographs, a description of which follows:

1. A stained preparation of the peritoneal exudate of a mouse two hours after the intraperitoneal injection of 0.01 cubic centimeter of a virulent culture of Type III Pneumococcus. The bacteria show well-defined capsules, and no evidence of phagocytosis is seen. Many polymorphonuclear and a moderate number of mononuclear leucocytes are present. (Gram stain. × 1000.)

2. A corresponding preparation of the exudate of a mouse two hours after receiving the same amount of culture together with 0.5 cubic centimeter of a preparation of the specific enzyme. The bacteria are devoid of capsules. Polymorphonuclear leucocytes predominate and phagocytosis is evident. (Gram stain. × 1000.)

3. A stained film of the peritoneal exudate of a mouse four hours after injection with 0.01 cubic centimeter of culture alone. The bacteria are increased in number, encapsulated, and extracellular. The cellular elements are polymorphonuclear and mononuclear leucocytes in about equal numbers. (Gram stain. × 1000.)

4. A corresponding preparation of the exudate of a mouse four hours after receiving the same amount of culture together with 0.5 cubic centimeter of a preparation of the specific enzyme. Marked phagocytosis has occurred and only an occasional organism is seen outside the accumulated leucocytes, nearly all of which are of the polymorphonuclear type. (Gram stain. × 1000.)

PROTECTIVE ACTION OF ENZYME IN MICE

Avery and Dubos then studied the protective action of the enzyme in mice against Pneumococcus Type III infection. By varying the amount of enzyme with a constant amount of culture, by keeping constant the dose of enzyme and by decreasing the quantity of culture, by giving the enzyme before and simultaneously with the infecting dose of culture, and by administering the enzyme by a single injection into mice eighteen hours after the onset of infection, the authors demonstrated that under the conditions

of the experiments, 0.1 cubic centimeter of the enzyme afforded mice protection against one million times the fatal dose of virulent Type III Pneumococcus. Avery and Dubos also showed that again the action of the enzyme was specific for Type III; that the greater the activity of the enzyme *in vitro* the greater was its protective action in mice; and that the enzyme exerted a curative action since the mice receiving the enzyme eighteen hours after the onset of infection recovered. The extraordinary enzymatic principle, therefore, not only strips the capsule from Type III Pneumococcus, but renders innocuous a large multiple of the infecting dose of the organism and, what is more, under certain conditions it actually cures mice of an already active infection.

PHILOSOPHICAL ASPECT OF THE ACTION OF THE ENZYME

The possibilities presented by this and other similar enzymes in the prevention and cure of pneumococcal infection in man were strikingly promising, but before going on to experiments leading in that direction, it may be interesting and more profitable to consider first the important philosophical aspects of the action of an enzyme of this particular type on bacterial polysaccharides. It would be impossible to improve on Avery and Dubos' discussion of this phase of the subject, so it is quoted in full.

The present study emphasizes the importance of the capsule in the biological reactions of the pneumococcus. It is, indeed, a significant fact, that no matter whether one regards this organism from the viewpoint of type-specificity, antigenicity, or its capacity to undergo variation, or whether, as in the present instance, one considers the pneumococcus with reference to its virulence and fate in the animal body, the one dominant factor influencing all these phenomena is the function of the cell to elaborate the specific capsular polysaccharide. These relationships, however, are not to be interpreted as meaning that virulence is dependent merely upon differences in the structural morphology of the bacterial cell. For it is a common observation that an encapsulated strain of Pneumococcus may be virulent for one species and not for another. However, it is equally true that the function of elaborating the

specific capsular polysaccharide is most highly developed in pneumo-
cocci that are best adapted to growth in the animal body. From this
point of view, virulence and capsule formation, although not causally
related, are at least intimately associated. When the function of forming
the capsular substance is suppressed or inhibited, as in the case of the
R variants, or when, as in the present instance, although this function
is unimpaired the capsule itself is destroyed by an enzyme, the naked
bacteria are thereby exposed directly to attack by the phagocytes of the
host.

In this sense, the action of the enzyme may be said to result in pre-
paring the encapsulated bacteria for phagocytosis; not as in the case of
antibodies, by specific sensitization, but by the process of decapsulation.
In the former instance, the reaction is an immunological one, whereby
the capsular material is altered by union with the type-specific anti-
body; in the latter case, the reaction is a chemical one in which the cap-
sular polysaccharide is actually decomposed by the enzyme. Although
the mode of action of both these specific agents is different in each in-
stance, the end result, so far as the fate of the microorganism is con-
cerned, is the same in both cases.

It is of interest that although neither the enzyme nor the specific
antibody is by itself bactericidal or bacteriolytic, yet each by reacting
specifically with the capsular substance exposes the virulent organisms
to the phagocytic action of the body tissues. The enzyme, like the spe-
cific antibody, serves merely to initiate the protective reaction, the com-
pletion of which is ultimately dependent for its successful issue upon
the effective cellular response of the host.

The present study also suggests that the capsule—long recognized as
a defense mechanism on the part of virulent bacteria—is a decisive fac-
tor in determining the fate of pneumococci in the animal body, and that
this structure is vulnerable to attack by specific agents other than anti-
bodies.

METHODS OF PRODUCTION

Dubos[335-6] then attempted the development of practical methods
of production, purification, and concentration of the enzyme, and
investigated the influence of certain factors on the potency and
primary toxicity of the preparations. He first studied the growth
and enzyme production of the SIII bacillus in more than fifty dif-

ferent media containing simple and complex saccharides, organic acids, alcohols, peptones, et cetera. Apart from the Type III specific polysaccharide he found only one other substance which caused the elaboration of the specific enzyme by the SIII bacillus and that was the aldobionic acid derived from the capsular polysaccharide of Type III Pneumococcus. When, however, he used another aldobionic acid, namely, that derived from gum arabic—a substance closely related immunologically to the Type III polysaccharide—no growth and, therefore, no enzyme could be obtained in the synthetic medium.

Dubos then learnt that the production of enzyme was also influenced by the concentration of capsular polysaccharide in the medium. The yield of enzyme rapidly increased with rising concentrations of the soluble specific substance, but decreased when the concentration exceeded 0.1 per cent and was inhibited by concentrations of 0.3 per cent or higher. Yeast extract, along with the soluble specific substance, stimulated enzyme production, but Dubos' first preparations in this medium proved to be toxic for experimental animals. Yeast extract in itself was not toxic and only when acted upon by cultures did it become so. However, Dubos was able to reduce the toxicity of the enzyme preparation by lowering the concentration of yeast extract to 0.03 per cent, the minimum compatible with good yields of enzyme.

Dubos then resumed his endeavors to produce a purer and more potent preparation of the polysaccharide-decomposing enzyme from the SIII bacillus, and in two communications (Dubos,[332] and Dubos and Bauer[338]) reported the results. It was first found that the yield of enzyme was proportional within certain limits to the amount of inoculum; that it increased with a rise in the concentration of capsular polysaccharide; and that 0.1 per cent of the carbohydrate represented the optimal amount for the purpose. After testing the influence of salt concentration in the medium on the metabolic activity of the SIII bacillus, on the basis of the collected data a method was devised which was described as follows

The bacteria are grown in a solution of 2 per cent casein hydrolysate (pH 7.0) at 37°C. and under conditions of strict aerobiosis; the cells from the 16 hour old culture, separated by centrifugalization, are re-suspended in small amounts of distilled water.

A medium is prepared consisting of 0.1 per cent capsular polysaccharide and 0.1 per cent NaCl in distilled water. This medium is distributed in 25 cc. amounts in large Erlenmeyer flasks (1 liter capacity) to provide for aerobic conditions, and each flask is inoculated with the cells recovered from 500 cc. of the culture of the SIII bacillus in the casein hydrolysate medium. The material is incubated for 12–18 hours at 37°C. and the cultures tested to ascertain the disappearance of the specific polysaccharide and the absence of contaminants. The cultures are now frozen and thawed repeatedly to secure the release of the endocellular enzyme.

The enzyme is ultimately separated from the cell debris by filtration. However, since the cell suspension is very viscous, it is first subjected to the following treatment. The cell suspension is made alkaline to pH 10.0 by the addition of sodium borate. Equimolecular concentrations of dibasic sodium phosphate and calcium chloride are then added to bring about a heavy precipitate of calcium phosphate which facilitates the clarification of the material by centrifugalization; (it had been established previously that the enzyme is not adsorbed on the calcium phosphate at alkaline reaction). The supernatant which contains all the enzyme in solution is now passed through a Seitz filter, then through a Berkefeld (V) filter. It is important to observe that the enzyme is completely adsorbed on the asbestos pad of the Seitz filter; this, however, can be prevented by washing the filter with nutrient infusion-peptone broth previous to filtration. After this treatment, the enzyme passes through the filter without loss. The potency of this filtrate is such that 0.002–0.004 cc. are required to decompose 0.01 mg. of the capsular polysaccharide under the conditions of the test.

Dubos found that the SIII bacillus grew abundantly in casein-hydrolysate medium, but in this medium the organism did not form any appreciable amount of the enzyme responsible for the decomposition of the polysaccharide. When, however, large numbers of the bacilli grown in the casein medium were re-suspended in a solution of the capsular polysaccharide which constituted the sole source of carbon, the specific carbohydrate substrate was rapidly

decomposed, and filtered autolysates of the cell suspension exhibited tremendously enhanced enzymatic activity. For a given amount of capsular polysaccharide decomposed in the new phosphate-yeast medium, the yield of enzyme obtained was much larger than that recovered by the former cultural methods. The production of the enzyme always failed when conditions in the substrate were unfavorable to cellular multiplication.

With Bauer, Dubos[338] then described an improved technique for concentrating and purifying the enzyme preparations. They filtered the solution, made in the manner just described, under fifty pounds pressure through collodion membranes of the type described by Bauer and Hughes.[90] The enzyme, under optimal conditions of filtration, passed through membranes of an average pore size of 10.6 mμ but was held back by pores having a diameter of 8.2 mμ. When the enzyme solutions were filtered to dryness through membranes of such porosity as to hold back the active principle, and when proper precautions were taken to prevent or minimize adsorption, such as the preliminary "greasing" of the collodion membrane with meat-infusion peptone broth, the enzyme could be completely recovered by immersing the membrane in distilled water or physiological salt solution.

EFFECT OF ENZYME ON INFECTION INDUCED IN RABBITS

In 1932, Goodner, Dubos, and Avery[536] studied the action of the enzyme on the dermal infection of rabbits induced with Type III Pneumococcus, employing the Type III strain virulent for rabbits previously described by Tillett. The culture was grown in rabbit-blood broth and its virulence was maintained for rabbits by frequent animal passage. Of the blood-broth culture 10^{-8} cubic centimeters given intraperitoneally sufficed to kill mice within ninety-six hours, and when given intradermally into rabbits 0.000,01 cubic centimeter caused death or a protracted disease of severe character. The enzyme preparations were, for the larger part, purified and concentrated by the method of Dubos. The intradermal infec-

tions were induced by the procedure devised by Goodner and already described in Chapter VI.

The authors found that the injection of adequate amounts of the specific enzyme twenty-four hours after infective inoculation brought about an early and complete cessation of the disease. The blood stream was freed of pneumococci and the organisms disappeared from the local lesion in the course of a few hours. Following the administration of the enzyme, the temperature at first rose, but fell within twenty-four hours to normal levels; the local lesion failed to spread and soon showed signs of healing. The results obtained by Goodner, Dubos, and Avery in a large series of infected animals indicated that in cases with severe bacteriemia large quantities of the enzyme were necessary to obtain successful results, while in animals having fewer organisms in the blood at the time of treatment smaller amounts of the enzyme would suffice.

The curative action of the enzyme was specific, at least in so far as it was tested. When given intravenously in a dose of one hundred units* twenty-four hours after inoculation with ten and one hundred minimal infective doses of Type I Pneumococcus, it failed to save the lives of the two rabbits tested. The authors found that the curative action of the enzyme was destroyed by heating at 70° for thirty minutes.

In a subsequent communication appearing later in the same year, Goodner and Dubos[535] reported the results of studies on the quantitative action of the enzyme on the dermal infection of rabbits with Type III Pneumococcus. With the same Type III culture and enzyme preparations similar to those employed in the previous experiments, using the number of pneumococci present in the blood as an indicator of the severity of the infection, Goodner and Dubos determined the amounts of enzyme required to bring about the recovery of the animal. It was ascertained that the required dose of enzyme bore a definite relation to the number of organisms circu-

* According to Dubos: "A unit of enzyme may be defined as one hundred times the smallest amount which will bring about the complete decomposition of 0.01 milligrams of the purified specific capsular polysaccharide in 18 hours at 37°C."

lating in the blood stream at the time of treatment. Thus, in animals with negative blood cultures or with a low grade bacteriemia, an amount of enzyme as small as five units might suffice to save the life of the animal; with a bacteriemia of 100 to 1,000 organisms per cubic centimeter of blood, twenty units might be necessary; with a bacteriemia of 1,000 to 10,000 organisms fifty units were required. In rabbits in which the bacteriemia exceeded 10,000 organisms per cubic centimeter a single injection of even one hundred units failed to rescue the animal, although infections of this order were successfully treated by repeated injections of large amounts of enzymes over a period of days.

Goodner and Dubos, in the discussion of their experimental data, stated that, although the quantitative relation established in the experiments was between the amount of enzyme and the number of pneumococci present in the blood, the fundamental relation was that existing between the quantity of enzyme and the total amount of specific capsular polysaccharide present in the body; and that an index of the latter was the degree of bacteriemia. The authors concluded that the enzyme is not a therapeutic agent per se, but one which, by decomposing the capsular substance of pneumococci and thus preparing the bacterial cells for phagocytosis—pianticating them, to use Friel's term—initiates a process which the body must be in condition to carry on if the animal is to recover. Hence, in the use of the enzyme, this capacity of the body must be reckoned with.

EFFECT ON INFECTION INDUCED IN MONKEYS

With this groundwork built, the next logical step was to apply the enzyme to the treatment of animals higher in the zoological scale which were suffering from experimental Type III Pneumococcus infection. Francis, Terrell, Dubos, and Avery,[477] accordingly, chose young adult monkeys—the Java monkey (*Macacus cynomolgos*)—as test animals, and infected the animals with a strain of Type III Pneumococcus virulent for rabbits. They employed the
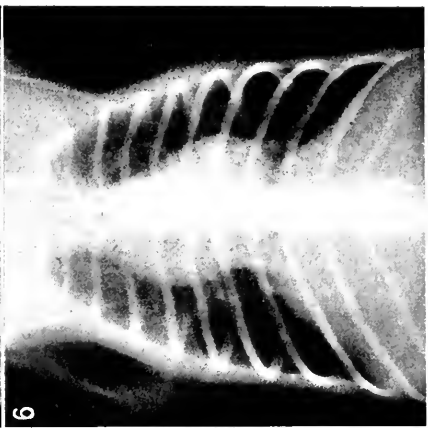
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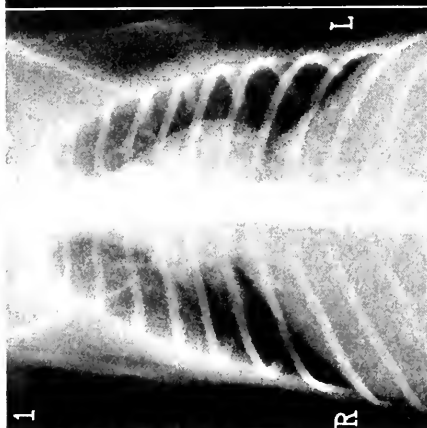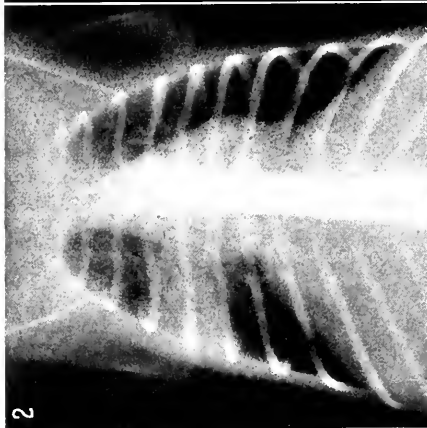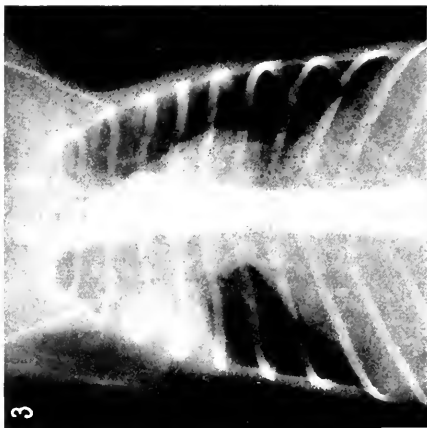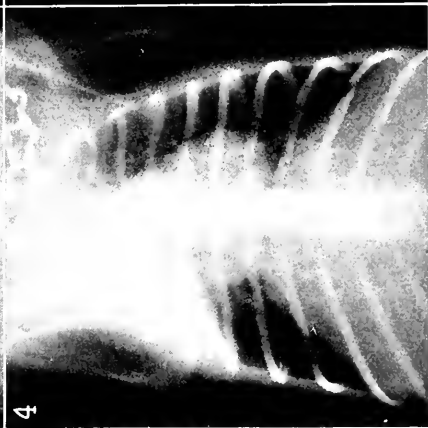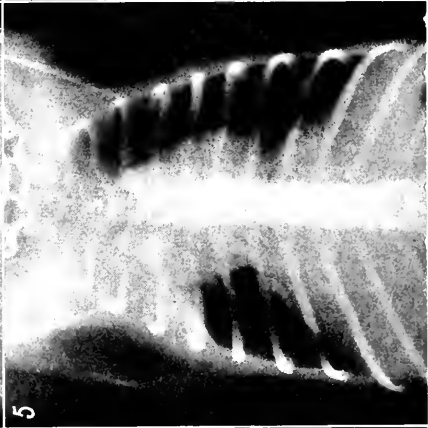
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After Francis, Terrell, Dubos, and Avery[57]

LUNGS OF A MONKEY DURING THE COURSE OF EXPERIMENTAL
PNEUMONIA TREATED THE FIRST DAY AFTER INFECTION

technique developed by two of them (Francis and Terrell[476]), who had found that by intratracheal or intrabronchial inoculation of Type III cultures it was possible to produce in monkeys of the *Cynomolgos* species an experimental pneumonia, which in its clinical aspects resembled pneumococcal lobar pneumonia in man. The authors used enzyme preparations made in the manner already described, which varied in potency from two to twenty units per cubic centimeter. They began the enzyme treatment within the first three days after inoculation, administering the enzyme preparations intravenously usually in doses of ten cubic centimeters each, with additional treatments as often as three times daily, either intravenously, intraperitoneally, or by both routes simultaneously. Francis, Terrell, Dubos, and Avery purposely made no attempt to ascertain the minimal amount of enzyme required in individual monkeys but, rather, they employed the treatment intensively until the result was assured. Moreover, they selected for enzyme treatment the sickest monkeys among those infected, since this plan would reduce the factor of natural recovery from the infection as well as put the enzyme to the severest test.

The accompanying photographs show the effect of pneumonia on the lungs of a monkey that had been treated the first day after infection, as follows:

1. Control. Before inoculation (December 19).

2. Nineteen hours after infection and seven hours before the first treatment (December 20), showing well localized consolidation in lower half of the right upper lobe.

3. Second day (December 21), showing extension of pneumonia throughout right upper lobe.

4. Third day (December 22), showing increased density of the shadow over the right upper lobe, but no evidence of further spread.

5. Fifth day (December 24). Resolution of the pneumonia has begun, as shown by the decrease in density and beginning aeration of the area.

6. Tenth day (December 29), showing complete resolution of the pneumonic shadow.

THE INFLUENCE OF ENZYME THERAPY UPON MORTALITY-RATE IN
EXPERIMENTAL TYPE III PNEUMOCOCCUS PNEUMONIA
IN MONKEYS*

Class of infection	Untreated animals				Treated animals			
	Total	Recovered	Died	Per cent mortality	Total	Recovered	Died	Per cent mortality
Pneumonia without septicemia	20	20	0	0	8	8	0	0
Pneumonia with septicemia (1–250)†..	20	11	9	45.0 ⎫	15	15	0	0 ⎫
Pneumonia with septicemia (250–2000)†	12	3	9	75.0 ⎬ 56.2	9	8	1	11.1 ⎬ 4
Pneumonia with septicemia (2000+)†	16	0	16	100.0	8	1	7	87.5
Total	*68*	*34*	*34*	*50.0*	*40*	*32*	*8*	*20.0*
Total for groups with septicemia	*48*	*14*	*34*	*70.8*	*32*	*24*	*8*	*25.0*

* Classified on the basis of height of septicemia in first three days.
† The numbers in parentheses in the left-hand column equal the cocci present in one cubic centimeter of blood.

The condensed results of experiments on forty monkeys are given in the accompanying table. The figures in the table represent only the recovery or death of the monkeys, and do not give in detail the effect of the enzyme on the course of the pneumococcal infection. As Francis, Terrell, Dubos, and Avery reported, it can readily be seen that in the groups in which no invasion of the blood occurred, spontaneous recovery was to be uniformly expected, whereas in the extremely severe forms of the disease the great majority of animals were too completely prostrated to respond to any therapeutic aids. Further comment of the authors on the action of the enzyme upon the infected monkeys can best be given in their own words:

In addition to the apparently beneficial effects of specific enzyme therapy as measured by survival or death of the animals, certain other favorable influences were observed. In a high percentage of cases in

which extension of the pneumonic process was occurring at the time of treatment, the spreading promptly ceased following the initial injection of enzyme. Although the density of the area of consolidation might at first appear greater than before treatment, extension did not occur and resolution of the lesion soon began. This limitation of spread of the pneumonia was not infrequently noted in the severe cases before the bacteria were completely eliminated from the blood stream. A comparison of the ultimate degree of pulmonary involvement in the treated and untreated cases reveals the fact that it was less, in general, in the former series. While in the treated cases the extension was apparently limited early, in the untreated animals extension of the pneumonia progressed, frequently with fatal results.

That the administration of enzyme promoted sterilization of the blood stream seems certain. In the milder cases this occurred quite rapidly. In animals in which the higher degrees of septicemia were present, there was rarely an increase, more regularly a prompt decrease in the number of pneumococci in the blood following the administration of enzyme. Even in cases which eventually terminated fatally, or in which extreme septicemia occurred early in the disease, cultures of the blood showed a marked reduction in the number of bacteria within 4 to 5 hours after the first treatment.

Simultaneously with limitation of the pneumonia, beginning resolution, and elimination of septicemia, a fall in temperature usually occurred. In fact, there was a tendency for the fever to subside concurrently with the cessation of pneumonic spread, even though septicemia still persisted. Although a marked leukopenia was comparatively frequent at the time treatment was begun, the number of leukocytes rose with the beginning of recovery.

In fatal untreated cases with septicemia, a high incidence of positive cultures was obtained from pleural or pericardial fluids at autopsy. In many instances frank empyema or pericarditis was present. In the treated cases with severe infections which resulted fatally, the incidence of these complications was also high. In recovered animals of the treated series, therefore, a frequency of suppurative complications equal to that of the untreated animals might be expected. The fact that the treated animals which survived recovered without suppurative sequelae suggests that enzyme therapy either prevented the development of empyema and pericarditis or was therapeutically effective even in the presence of these complications.

As previously stated, many technical difficulties have been encoun-

tered in attempting to produce enzyme preparations of uniformly high therapeutic activity and purity. The different lots of enzyme have, as a result, been inconstant in both these respects. In some instances toxic effects, attributable to impurities in the material, have been noted in animals after the administration of enzyme. These impurities may induce a febrile reaction and a decrease in the white blood count of the animal. At other times, when the animal is extremely ill with subnormal temperature and a marked leukopenia, the administration of impure preparations may produce a further depression of temperature and of the leukocytes.

The results of the present study indicate that the specific enzyme, even in its present state of purity, exerts a favorable therapeutic effect upon the course and outcome of experimental Type III pneumococcus pneumonia in monkeys. Nevertheless, the present study again emphasizes the therapeutic limitations of the enzyme. The action of the enzyme is known to be exerted upon the capsular polysaccharide of Type III Pneumococcus. By being deprived of its capsule, the bacterium is made susceptible to phagocytosis by the cells of the animal body. However, when the disease process is of extreme severity and the entire cellular mechanism of the body is markedly depressed, the animal may no longer possess the capacity to dispose of the organisms rendered vulnerable by the specific action of the enzyme.

ISOLATION AND STUDY OF OTHER POLYSACCHARIDE-SPLITTING ENZYMES

In 1933, Sickles and Shaw[1281] discovered microorganisms, other than the SIII bacillus of Avery and Dubos, capable of decomposing the capsular polysaccharide of Type III Pneumococcus and, furthermore, succeeded in obtaining from soil another organism possessing an enzymatic action on the capsular polysaccharide of Type II Pneumococcus. From decaying vegetable matter from different localities, Sickles and Shaw isolated in pure culture three strains of sporulating bacilli, which in morphology and some of their other characters differed from one another and from the SIII bacillus. The organisms were all aerobic, Gram-negative, motile rods with peritrichous flagellae, and formed oval spores wider than the vegetative cells. On beef-extract agar the organisms grew in

colonies about two millimeters in diameter, which were yellowish white, smooth, and round with entire edges. On blood agar, two of the strains produced two types of colonies, one whitish and opaque and the other grayish and semi-translucent. One of the strains isolated from material from a decayed hickory stump covered with sphagnum moss appeared to utilize agar as well as the Type III polysaccharide. The cultures fermented dextrose, lactose, saccharose, maltose, dextrin, mannitol, xylose, galactose, inulin, and salicin as well as decomposing the specific polysaccharide of Type III Pneumococcus.

Sickles and Shaw made their enzyme preparations in a mineral substrate, using the yeast extract medium of Dubos only for the seed culture. The cultures were usually incubated for three days at 36°, filtered through a Berkefeld candle, and then concentrated by ultrafiltration through a 7.5 per cent pyroxyline membrane, the enzyme remaining on the membrane. In this manner a preparation was obtained with an enzymatic activity of approximately twenty units per cubic centimeter. The enzyme in a dose of 0.5 cubic centimeters, when given eighteen hours after inoculation, protected mice against infection with ten minimal fatal doses of Type III Pneumococcus and, when given seven hours after inoculation, the extract protected the animals against 1,000 fatal doses.

The organism isolated by Sickles and Shaw from soil, possessing the power to decompose Type II soluble specific substance, differed markedly from the bacillus attacking Type III SSS. It, too, was an obligate aerobe, but oval in form, resembling a yeast. It could be maintained by daily transfer on the mineral medium containing 0.01 per cent Type II soluble specific substance, but no growth could be induced in the mineral medium alone or on any of the usual nutrient solid media. No growth could be seen in meat-extract broth but microscopic examination indicated that slight multiplication had taken place. The organism grew slowly in meat-extract broth containing a fermentable carbohydrate, but did not grow in meat-infusion broth, litmus milk, or peptone water, on po-

tato slants, or in gelatine. Growth was similarly slow on a solid me-
dium composed of agar and the mineral medium containing Type
II polysaccharide. In the mineral medium to which one per cent of
carbohydrate was added, some strains of the organism fermented
maltose, xylose, and dextrin, while all strains decomposed lactose
and saccharose.

The cultures broke down the Type II cellular carbohydrate of
Wadsworth and Brown as well as the soluble specific substance of
the same type, but failed to dissolve the specific polysaccharides
of Type I and III pneumococci. In the case of this organism, how-
ever, the enzyme was active only when the body of the organism
was present, but Sickles and Shaw apparently did not test filtrates
from the cultures. At that time no experiments were performed on
the protective properties of this culture. It was observed, how-
ever, that the organism in decomposing the cellular carbohydrate
robbed it of its purpura-producing property, but this effect may
have been due to the digestion of admixed protein material of pneu-
mococcal origin by a proteolytic ferment in the enzymatic prepa-
ration employed.

In a later paper, Sickles and Shaw[1283] reported the isolation
from soil of another organism—a small Gram-negative bacillus—
which acted on the Type I specific carbohydrate but failed to ef-
fect a complete decomposition of that substance, since it never
fully lost its precipitating ability with homologous serum. Sickles
and Shaw suggested that this residual reaction was due to an un-
used portion of the original carbohydrate or to products of de-
composition, which might either be present in the original sample
or be formed as a result of the action of the microorganism from
the soil. Another possibility presents itself and that is that the au-
thors' carbohydrate preparation may have contained a fraction
which was not susceptible to the enzymatic action of the culture.
It was impossible to obtain any soluble enzyme from this organism.

Several of Sickles and Shaw's cultures decomposed the non-
type-specific carbohydrate isolated by Wadsworth and Brown (the

C Fraction) from an attenuated strain of Type I Pneumococcus. One organism was the aerobic, spore-forming bacillus that had been found to utilize the soluble specific substance of Type III Pneumococcus and agar; the other was a Gram-negative aerobic, non-motile, non-sporulating bacillus which, so far as tested, digested only this non-type-specific saccharide. From both of the strains a soluble enzyme was obtained capable, like the living cells, of splitting the non-type-specific carbohydrate and, in doing so, of abolishing its power to produce purpura.

Sickles and Shaw[1282] then undertook a systematic study of the morphological, cultural, and biochemical characters of these various polysaccharide-decomposing bacteria. The cultures had originated in the muck of swamps and in uncultivated soils of different localities, while one strain came from manure. The organisms were grown on the mineral medium of Dubos and Avery and a "Medium S" of their own concoction. To these mineral media were added the specific pneumococcal carbohydrates in concentrations varying from 0.002 to 0.01 per cent as a source of carbon.

Sickles and Shaw then subjected the strains to all the procedures required for identification, and in their communication gave a detailed account of the different biological characters of the organisms. To relate them would not be germane to the main subject but, because of the importance of this class of bacteria, a condensed description of the main characters should be recorded here. The cultures were of four distinct types:

1. Large spore-bearing rods that decompose the specific carbohydrate of Type III Pneumococcus, similar to the SIII bacillus of Dubos and Avery, and, in addition, a strain that utilizes agar. For the strains that digest only the Type III polysaccharide the authors suggested the name *Bacillus palustris,* with the sub-designation, *gelacticus,* for the variety also attacking agar.

2. Very small non-sporulating rods that attack the non-type-specific carbohydrate obtained from a degraded Type I Pneumococcus and the C Fraction from typical strains. This organism the authors called, *Flavo-bacterium ferruginum.*

3. Bacteria, oval in form, that decompose the specific carbohydrate of Type II Pneumococcus. To these organisms was given the species name of *ovale* and the designation *Saccharobacterium* was proposed for this new genus of the family *Mycobacteriaceae* including the microorganisms decomposing the specific carbohydrates of Pneumococcus of Types I and II.

4. Slender rods with tapering ends that utilize the specific carbohydrate of Type I Pneumococcus, for which the authors suggested the name *Saccharobacterium acuminatum*.

Again from soil, Sickles and Shaw[1284] isolated another strain of *Bacillus palustris*, which through the agency of a soluble enzyme decomposed the specific carbohydrate of Type VIII Pneumococcus. The enzyme was apparently without action on organisms of Types I, II, or III. Although Type VIII specific carbohydrate gave marked precipitation with Type III antiserum and, conversely, Type III carbohydrate precipitated Type VIII antiserum, it was a curious fact that the culture or enzyme that decomposed Type III carbohydrate failed to attack the polysaccharide of Type VIII, and the enzyme decomposing Type VIII carbohydrate was without action on the carbohydrate of Type III pneumococci. Here the enzymatic selectivity appears to be even greater than the overlapping serological specificity exhibited by chemically related but not structurally identical carbohydrates.

DIFFERENCES IN SUSCEPTIBILITY OF POLYSACCHARIDES TO ENZYMATIC ACTION

A possible explanation for the differences in the susceptibility of pneumococcal polysaccharides to enzymatic action and in their serological behavior is to be found in the recent study of Goebel (1935).[519] Quoting from his communication:

In the present study the specific polysaccharide of Type VIII pneumococcus has likewise been shown to be constituted from molecules of glucose and glucuronic acid. After hydrolysis with dilute mineral acid there appear in the hydrolysate of the carbohydrate from Type VIII

pneumococcus a hexose, identified as glucose, and an aldobionic acid. From the actual quantities of these two constituents found in the hydrolysate, and from the value of the acid equivalent of the polysaccharide itself, it appears that this specific carbohydrate is built up from glucose and glucuronic acid approximately in the ratio of 7 molecules of hexose to 2 of uronic acid. The capsular carbohydrate of Type VIII pneumococcus represents, therefore, an entity which is chemically distinct from the specific polysaccharide elaborated by the Type III pneumococcus.

That a chemical similarity between the two substances exists, however, may be seen from the results of the experimental work in which it has been shown that the aldobionic acids appearing in the acid hydrolysates of both polysaccharides are identical. The proof of the identity of these two uronic acids was made possible through the preparation of the crystalline heptaacetyl methyl ester. Both derivatives show identical crystalline structure, melting points, and specific rotations. Furthermore, a mixed melting point of the two derivatives shows no depression. Although the actual structure of the aldobionic acid is as yet unknown, work is now in progress to establish this point.

In view of the experimental evidence which has been presented, showing that the aldobionic acids derived from the carbohydrates of Types III and VIII pneumococcus are identical, it is believed that the basis for the immunological crossing exhibited by these two specific types of pneumococcus resides in the structural and configurational identity of the uronic acid nucleus common to the encapsulating polysaccharides of both microorganisms.

Sickles and Shaw[1283] reported that the enzyme from the new soil bacillus was capable of destroying the purpura-producing action of the VIII carbohydrate and of protecting mice against several thousand minimal fatal doses of a virulent strain of Type VIII Pneumococcus. Larger amounts of the enzyme failed to protect mice against ten minimal fatal doses of Type III Pneumococcus. The observations in these two papers describing the highly selective action of the enzyme on the soluble specific substance in contrast to the cross-agglutination present another pretty problem for the chemo-immunologist.

SUMMARY

The discovery of bacteria endowed with enzymes capable of breaking down the complex polysaccharides of Pneumococcus is of more than academic interest. Here is a biological agent that in no way interferes with the vital activities of the pneumococcal cell; the cell grows, multiplies, and continues to synthesize the complex capsular carbohydrate peculiar to its serological type, but as soon as the carbohydrate is formed it is stripped from the coccus by the digestive action of the alien bacterial enzyme. The ability of a mere saprophyte to rob a pathogen of its protective covering, and thereby to deprive it of its infective power, is one of the strangely fascinating surprises of bacteriological research, but it has a much deeper meaning. The nature of this enzymatic action is in accord with the specific chemical constitution and serological behavior of the specific capsular polysaccharide of the pneumococcal type affected. By denuding the cell of its capsule through the dissolution of its polysaccharide, the enzyme exposes the cell to the destructive action of the white corpuscles of the blood, and if the tissue cells are functionally vigorous enough, the attack of the invading pneumococci is repelled and the infection aborted.

The enzymes of certain of these bacterial forms have been shown to save mice, rabbits, and monkeys from all but a massive infection from Pneumococcus and, what is more striking, the enzyme of one bacterial species directs its attack against the members of Type III, for which, as yet, no biological curative agent has been devised. The possibilities presented by these recent disclosures engage the imagination. The bacteriologist will want to search for still other microbic cells with enzymes capable of consuming the carbohydrates of pneumococci of the other serological types; the biochemist will desire to know more of the nature of the enzymatic principle existing in these bacterial cells; while the investigator in immunology will follow this inviting lead toward a resolution of some of the processes operating in infection and resistance.

ANTIGENICITY OF PNEUMOCOCCUS

*The immunizing properties of Pneumococcus and of its compo-
nents and derivatives; factors which operate in establishing the im-
mune state; and the response of different animals to the antigenic
action of members of this bacterial species and their constituents.*

IN Chapter VIII the antigenic properties of the chemical con-
stituents of Pneumococcus were described only in a general way
in order to illustrate the similarities and dissimilarities of the pro-
tein and carbohydrate preparations which have been advanced as
representing the active substances native to the cell, and to empha-
size the dependence of immunological behavior on chemical consti-
tution. No attempt was made to give any systematic account of
the various antibodies or immune effects which the proteins and
polysaccharides call forth, or to compare and correlate the action
of the cellular fractions with that of the intact bacterial body.

Antigenic Spectrum

The antigenic spectrum of Pneumococcus can be resolved into
its dominant bands in several ways. Taking the whole, living, fully
virulent cell as the norm, its immunological action can be analyzed
through the stimuli provided by its separate constituents or de-
rivatives. There enter into the analysis the chemical structure of
the several components of the cell, the question of their mass, and
the route and spacing of their administration.

The spectral bands—or less definitely, zones or regions—ap-
pearing after the introduction of pneumococcal materials into the
animal body, are represented by agglutinins, precipitins, bacteri-
cidins, tropins or opsonins—conceivably the alleged antitoxins—
the complement-fixing and protective antibodies, and such manifes-

tations of somatic reactivity as systemic or local allergy. All the phenomena depend in turn on the inherent special or racial peculiarities of the animal subject.

The resolution of the antigenic spectrum involves the study of the effects of Pneumococcus itself, of its separate constituents with their individual, chemical identification, in their relation to the parent material; the diverse serological reactions in which the whole cell or its parts participate; and the nature of the mechanism of the particular physiological response each wakens.

These ways bristle with obstacles of one kind or another. There is the tendency of Pneumococcus to digest itself or to lose its vigor; there is the danger of fracturing the molecular mosaic of the cellular substances by chemical treatment; there are all the subtle factors which influence the interaction of antigen and antibody in the test tube; and then there is the constitutional capacity or incapacity of animal tissues to function in response to the introduction of alien substances into the body. An antigen by one test may display full power; by another test it may seem to be incomplete or inert. Introduced in inappropriate quantity, an antigen may defeat its effect; administered by one route we see one set of immune bodies, by a different route some of the antibodies may appear wanting type-specificity or else be entirely absent; injected into animals of one species an antigen provides the animal with a full complement of antagonistic substances and protects it against fatal infection, while in other animals the administration of the same antigenic substance may be followed by no appreciable immune effect. Here, not only the race but the lineage, the age, and the immediate physical state of the animal all have their part in deciding the issue. Antigens and antigenicity, like virulence, therefore, are purely relative terms.

First Observations of Immunity

For a complete and orderly presentation of the manifestations of the immunological behavior of Pneumococcus it seems best to

develop the subject by showing the manner in which our present knowledge has been acquired. Although it was Fraenkel[468] who first observed that rabbits surviving a subcutaneous injection of pneumococci became resistant to subsequent inoculation with the same organism, it was Foà and Bordoni-Uffreduzzi[461] who established the fact in an experimental way. By injecting rabbits subcutaneously at three or four-day intervals, beginning with attenuated cultures and then giving cultures of increasing virulence, the animals became insusceptible to inoculation with virulent cultures or infected blood. The authors applied the method to man but with no success. Then Foà[458] turned to the soluble products of Pneumococcus, and attempted the chemical isolation of the immunizing principle from broth cultures, but the experiments were inconclusive.

A great debt is owing the Klemperers,[723-5] who laid the foundation for the active immunization of animals against pneumococcal infection. As has already been told, the Klemperers used the sputum of convalescent pneumonia patients, bacteria-free pleural exudates, heated glycerol extracts, and heat-killed cultures as antigens and hence became the first to demonstrate that dead pneumococci and some of their derivatives, quite as well as the living cells, can render an animal immune. They also introduced the intravenous route for the administration of antigenic materials and proved that the serum of the immunized rabbits carried substances antagonistic to Pneumococcus. Like Foà, the Klemperers believed incorrectly that the immunizing principle in Pneumococcus was a toxin.

There then began a succession of publications dealing with the action of various pneumococcal materials in inducing immunity. There were the successful results of Emmerich and Fowitzky[357] with cultures of attenuated and then of diluted, virulent strains, and those of Bonome,[137] and of Kruse and Pansini,[763] with sterile culture filtrates, injected intravenously, subcutaneously, and intraperitoneally. Mosny[932] was another to employ sterile culture fil-

trates, while Foà and Carbone[463] obtained a certain degree of immunity with alcohol and ammonium sulfate precipitates from culture filtrates. The antigenic action of these agents was feeble, but Foà and Carbone demonstrated that both the intact cell, living or dead, and substances elaborated by the cell during cultivation evoke an immune response. The work of Denys[312] and of Washbourn[1486] established the fact that in order to produce immunity of high grade it was essential that pneumococci, whether used in the living or dead state or in the form of heated or filtered cultures, should be virulent.

These were the beginnings of immunizing procedures which have become routine, but there are many features of the subject that deserve more detailed discussion. The use of pneumococci taken from growths on solid media or in broth for the production of immune serum for experimental purposes has long been the common custom. For some time it was believed that for the production of a potent serum it was necessary to inject living organisms, but experience has shown that response to antigenic stimulus, that is the production of a serum with a high content of strictly type-specific antibodies, is conditioned less by the viable state of the organism than by the biological state of the culture at the time it is employed as antigen.

Influence of Virulence on Immunological Response

It is now generally accepted that for the production of a high degree of type-specific immunity the antigen, whether in a living or devitalized condition, must come from a robust, virulent culture. This conviction arises from the results of experiments on animals with strains possessing varying degrees of invasiveness, from the fully virulent, smooth forms to the degraded, rough forms, and from the experience of those workers who are engaged in the manufacture of therapeutic serum. The reason supporting the conviction is the chemical demonstration that complete antigenicity is

dependent upon the maximal amount of unaltered protein and car-
bohydrate in the cell.

All through the literature on pneumococcal immunity, from the
time of Denys and of Washbourn, there are repeated statements to
the effect that virulent pneumococci are more active immunizing
agents than are attenuated strains. The study of the antigenic ac-
tion, especially in regard to type-specificity, of variant forms of
pneumococci substantiates this fact. Neufeld, Cole, Wadsworth,
and others, have always advocated the use of cultures in a virulent
state for the production of immune serums. Barach[75] has shown
that a highly virulent pneumococcus provokes a more marked im-
munity than an organism of low virulence—an observation cor-
roborated by Meyer and Sukneff,[898] Day,[308] and many others.
Therefore, whether the immunizing antigen is to be the living or
killed organism or any of the separate substances isolated or de-
rived from the cell, the evidence is wholly in favor of selecting a
culture in vigorous condition and of exalted virulence.

Dead Cultures

HEAT-KILLED ANTIGENS

For a long time living pneumococci, usually after a preliminary
course of injections of heat-killed cultures, were used for the vac-
cination of horses in the production of curative serums. The prac-
tice, however, caused a grave mortality among the animals under
treatment. In 1917, the senior author of this volume, among
others, desiring to prevent the depletion of his stables, substituted
for living cocci, heat-killed organisms from broth cultures, and al-
though the agglutinin titer of the horses was low, the content of
the serum in mouse-protective antibodies was more than sufficient
to meet standard requirements of that time.

Using cultures devitalized by heat, formaldehyde, soaps, or
other means became the practice in many serum-producing labora-
tories, although the literature contains relatively few detailed ob-

servations on the comparative antigenic action of pneumococci when in the living and in the dead state.* In 1902, Neufeld[974] obtained satisfactory agglutinating serum by the injection of rabbits with either living or heat-killed pneumococci, but for the routine preparation of immune serum he gave the animals, first, heat-killed cultures, and then living cultures. Wadsworth,[1457] in 1912, claimed that only after immunization with living, virulent cultures did the serum acquire marked curative properties.

Cotoni and Brasic[281] made a comparative study of the immunizing effect of pneumococci heated at 56°, of cultures treated with alcohol and ether and suspended in salt solution, and of these suspensions heated for fifteen minutes at 110°. The heat-killed cocci proved to be far superior to the other antigens in protecting rabbits against infection.

The influence on antigenic action of varying exposures of pneumococci to different temperatures was investigated by Tani.[1379] According to the results obtained, cultures of Type I pneumococci incubated for a long period at 39°, when injected intraperitoneally into mice, were found to have lost virulence and were feeble immunizing agents; cocci heated for two and one-half hours and not completely killed showed poor immunizing power, whereas another specimen heated at the same temperature for an equal length of time and completely killed, and then later heated for one-half hour at 56°, gave good protection. After subjecting a similar culture to a four hours' exposure at 100°, it was still antigenic in that it afforded protection to mice. Tani's results point to virulence as an essential property of cultures used for immunization. Davidson,[298] too, tested the antigenic action of living pneumococci, of heat-killed cultures, and of detoxified and defatted vaccines. Rabbits injected with the killed cultures acquired immunity, and the serum contained protective substances. The living cultures pro-

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* These facts and many similar observations by others have never been published but are widely current in the stable lore of manufacturing laboratories.

duced intoxication, while the use of acetone and other agents in removing the lipids from the vaccine destroyed its antigenic power.

In the case of Type III Pneumococcus, Tillett[1405] found that repeated injections of heat-killed cultures into rabbits were effective in producing active immunity against infection with a virulent strain of the homologous type. Even heterologous strains, devitalized by heat, were capable of affording similar protection to Type III cocci. Moreover, this form of active immunity could exist in the absence of demonstrable type-specific antibodies and apparently was unrelated to the variety of Pneumococcus used for immunization. Tillett considered that this anomalous immune state was dependent upon the exaltation of the same factors which afford normal rabbits material resistance to some strains of Type III organisms.

Employing Type II strains, Gaspari, Sugg, Fleming, and Neill[503] learnt that the continued injection of heat-killed cultures of this type led to a decrease in type-specificity and to an increase in the species-specificity of the serum of the treated rabbits. For the preparation of diagnostic serum of high type-specificity the authors preferred as an antigen the heated cells of virulent pneumococci administered over a comparatively short immunization period. A gradual shift from type-specificity to species-specificity is no uncommon occurrence in horses that have been under immunization for long periods of time with either living or dead organisms.

Day[307] reported that it was generally desirable to kill the cells with heat for the production of antibodies, but warned against the use of a temperature as high as 100° for the purpose, since such a degree of heat destroyed the antigenic properties of the culture. However, this has not been the experience of the authors of the present volume. As might be expected, Day, and also Harley,[592] found that any agent which promoted the digestion of the cell, and which consequently led to the breakdown of its constituents, weakened or finally abolished the ability to call forth type-specific anti-

bodies. Additional evidence confirming the efficacy of heat-killed antigens is to be found in the experiments of Stillman and Goodner.[1342] Three intravenous injections at four-day intervals of saline suspensions of heat-killed cultures of Type I, II, and III pneumococci protected rabbits against an otherwise fatal infection of pneumococci of the corresponding type administered according to Goodner's intradermal technique. The results were controlled by the demonstration of agglutinins and protective antibodies in the serum of the treated animals.

In 1923, Yoshioka,[1562] finding that the successful immunization of mice was largely dependent upon the total mass as well as the spacing of the doses of antigen, devised a rapid method involving small quantities of heat-killed pneumococci injected in six half-hourly doses. In this way he obtained excellent protection in mice. In the next year, Killian[704] repeated Yoshioka's experiments with intensive serial injections and, in addition, tried injections of killed cultures with weekly intervals between each series. By this plan of three series of intraperitoneal injections at weekly intervals, it was possible to render mice immune to a fatal dose of pneumococci given three days after the completion of the immunizing treatment, but the immunity lasted only a few weeks. Killian laid stress on the proper amount of antigen to be given, because doses too small or too large yielded poorer results. The use of living, fully virulent organisms, contrary to the experience of most observers, resulted in a noticeably lower degree of immunity.

In another communication, Killian[706] gave the details of further experiments from which it appeared that by employing the intravenous route for serial injections the level of protection in mice could be elevated, and that by periodic intraperitoneal treatment of the animals with four-day intervals between injections of increasing doses of killed vaccine one could obtain a high degree of protection in a short time. In a third communication, Killian[707] described a refractory stage which lasted for several days after an immunizing injection and, in order to avoid this stage and to se-

cure the most effective secondary stimulus, gave injections at intervals of fourteen instead of four or eight days.

The question of the effect of heat on the immunizing activity of Pneumococcus was subjected to systematic study by Barnes and White,[86] who compared the antibody response following the injection into rabbits of heat-killed and formalinized cultures of Type I Pneumococcus, administered according to different schedules over long periods—in some of the experiments for as long as eight months. As antigens, the authors used saline suspensions of the sediment from eighteen-hour broth cultures of a virulent Type I strain, subjected to 56° in a water-bath for one hour, and similar suspensions of culture sediment treated with 0.3 per cent formalin. The vaccines were standardized by the Gates[504] nephelometer and the individual doses accurately measured. The injections were given intravenously according to three different plans somewhat similar to those of Yoshioka and of Killian. The schedules consisted respectively of a single injection on each of three successive days; a single injection on each of five successive days; and a series of six injections on each of the first two days with the other four on the third day. The rest periods varied from one to three weeks, and test bleedings were usually taken seven, twelve, and, in some instances, twenty-two days after the last injection of a series. The various serum samples were titrated for agglutinins, precipitins, and mouse-protective antibodies.

Heat-killed pneumococci produced highly potent serums of strict type-specificity. The first and third named methods were equally productive, but the first was the method of choice because of its greater simplicity. The results of the study, besides confirming the value of heat-killed pneumococci in establishing a high degree of immunity, afforded a rational basis for the immunizing treatment of horses for the production of therapeutic serum.

OTHER DEVITALIZED ANTIGENS

As soon as it was found that heat-killed pneumococci were capa-

ble of calling forth a specific immune response, other attempts
were made to rob the cell of the power of invasion and infection
without impairing its antigenic qualities. For a long time for-
maldehyde in the form of formalin has been employed for the pur-
pose of killing and preserving pathogenic organisms, but it is not
clear when it was first used to prepare pneumococci for the pur-
pose of immunization. Tao (1932)[1380] reported that the antigenic
potency of formalinized cultures was greater than that of heat-
killed cultures in causing the development of agglutinins and pro-
tective antibodies in rabbits and mice. Formalin in a concentration
of 0.3 per cent was added to the cultures and the treatment of the
animals was limited to one subcutaneous and two or three intra-
venous injections.

Pico and Negrete[1089] prepared immunizing antigens for horses
with 0.05 per cent formalin. The authors attributed the keeping
qualities of the vaccine to the action of formaldehyde in rendering
the soluble specific substance insoluble in water, but it has been the
experience of the writers of the present volume that concentrations
of formalin as high as 0.3 per cent fail to prevent the gradual lysis
of pneumococci suspended in salt solution. In Ferguson's[436] experi-
ence in actively immunizing white mice and rabbits with both heat-
killed and formalinized vaccines, it appeared that the latter were
only slightly superior to heat-killed cultures. Formalin was used in
a strength of 0.2 per cent, and suspensions were made in salt solu-
tion containing 0.5 per cent phenol. There was a tendency of killed
pneumococci to autolyze with a loss of antigenic power accom-
panying the disintegration of the cell. For this reason, as well as
because of their better immunizing effect, Barnes and White gave
their choice to heat-killed vaccines, since these vaccines undergo
less deterioration than those prepared with formalin.

According to Dubos,* formalin added in a concentration of 0.3
per cent to suspensions of living pneumococci prevents multiplica-
tion but fails to inhibit the activity of intracellular enzymes. The

* Personal communication, 1936.

concentration of formalin must be raised to 0.5 per cent if enzymatic activity is to be destroyed. On the other hand, exposure of the pneumococcal suspensions to heat, when properly carried out, yields a vaccine of great stability and of high antigenic integrity. Dubos observed that exposure to temperatures that rob the cell of its ability to grow and multiply may fail to halt the action of the protease of Pneumococcus. To devitalize the organisms, to prevent autolysis, and to preserve the antigenic properties of pneumococci, Dubos heated cultures or suspensions to 75° with the least possible delay and so obtained antigens of greater stability and immunizing power than could be produced by any other method.

Confirming the claims of Barnes and White and of Dubos that immediate heating of pneumococci for use as antigens is the best method for insuring the stability of the preparation, O'Meara and Brown,[1031] during the course of an investigation on the readiness with which pneumococci disintegrate, reported that, in spite of a large number of preservatives tried, no agent except heat was found that would completely arrest autolysis of pneumococcal cultures.

Day[307] used formol, or antiformin, as a killing agent, but abandoned its use since it weakened the immunizing properties of the cultures. Truche[1419-20] favored an alcohol-ether mixture for the routine preparation of antigens in the production of equine antipneumococcic serum, and claimed that cultures so treated, dried in a vacuum, and suspended in salt solution, were tolerated better by horses than were heat-killed cultures.

There have been reported many other methods for rendering pneumococci harmless for immunizing purposes. As early as 1902, Sergent[1256] treated suspensions of agar cultures of pneumococci with crystal violet. Although the dye in the concentration used failed to kill the cocci it effected a partial attenuation, so that rabbits surviving the intravenous or intraperitoneal injection of the treated cultures were able six to eight days later to resist increased doses of the same cultures that proved fatal in animals which had

not received a previous injection of the dyed cocci. Sergent's application of coal-tar dyes to pneumococci for the purpose of preparing immunizing antigens appears to be a lone observation of its kind.

Nicolle and Adil-Bey (1907)[1007] obtained immunity in rabbits by the injection of cultures treated with sodium choleate, while Meyer (1927)[897] and later Meyer and Sukneff (1928)[898] reported that Type I pneumococci dissolved in sodium taurocholate would immunize both rabbits and mice. The serum of the rabbits so treated contained a moderate amount of protective substance but, as might be expected, the serum as a rule gave some cross-protection against Type II strains. While pneumococci treated with bile-salts may induce immunity in mice and rabbits and presumably in other animals, the immunity is of a low order and is lacking in type-specificity. As late as 1933, Ziegler[1573] advocated as an immunizing agent "Pneumocholin," a substance of unknown chemical composition produced by the lysis of pneumococci in sodium taurocholate solution. The substance was stable under refrigeration and, when injected into rabbits, induced after three or four days an effective immunity to Type I infection.

Larson and Nelson[791] recommended the use of sodium ricinoleate in the preparation of pneumococcal vaccines both for prophylactic use in man and for the preparation of therapeutic serum. A virulent culture of Pneumococcus treated with ricinoleate soap in a final concentration of 0.1 per cent immediately lost its pathogenicity and, according to the authors, when injected intraperitoneally into rabbits, stimulated the production of large amounts of agglutinins within twenty-four hours. The animals resisted many multiples of the infective dose of pneumococci, and their serum protected normal rabbits against both intravenous and intraperitoneal infection. The serum of rabbits and sheep immunized with the sodium ricinoleate antigens was claimed to possess distinct curative properties for rabbits infected with Pneumococcus. The administration of the immune serum to patients suffering

from lobar pneumonia was followed by a rapid drop in tempera-
ture and relief of subjective symptoms. Larson believed that the
effect was due to the presence of antitoxin in the serum.

In a later communication, Larson[789] reported that the intraperi-
toneal injection of large amounts of soaped pneumococci of Types
I, II, and III protected rabbits against infection induced by the
Goodner technique, and claimed that by this method it was possi-
ble to produce both species-specific and type-specific immunity.

Sensitized Pneumococci

The use of pneumococci sensitized with homologous immune se-
rum was described by Levy and Aoki (1910),[801] while Alexander,[7]
after similarly treating and incubating the cocci with leucocytes,
could immunize rabbits with the preparations. Protective sub-
stances appeared in the serum of the animals within eight to eleven
days after the first injection, but the method possessed no advan-
tages over the use of heat or formalin for the preparation of anti-
gens. Levy and Aoki, and also Killian,[704] employed phenol as a
devitalizing agent, but the latter author found that phenolized
vaccines showed a definite loss of immunizing power after six weeks'
preservation.

Filtrates and Extracts

CULTURE FILTRATES AND BACTERIAL EXTRACTS

Early investigators turned to culture filtrates and to other
products and derivatives of Pneumococcus in a search for some
principle which would increase the resistance of experimental ani-
mals and eventually of man to pneumococcal infection.

Warden (1912)[1485] claimed to have immunized rabbits with pan-
creatic extracts of pneumococci and made the additional observa-
tion, which apparently has never been confirmed, that similar ex-
tracts of staphylococci and pancreatic extracts alone were capable
of inducing immunity in infected and non-infected rabbits, and of

favorably modifying the course of pneumococcal infections in man. However, in Warden's communication no experimental data were presented to support these unusual claims. Aïtoff and Lagrange (1925)[6] stated that an injection of sterile medium T made at least twenty-four hours previous to inoculation with Pneumococcus conferred protection upon mice which was demonstrable for at least two weeks after injection of the medium.

In 1926, Horder and Ferry,[655] in a search for an ideal antigen for therapeutic immunization, measured the agglutinin content and complement-fixing titer of the serum of rabbits treated respectively with washings from agar cultures, autolysates of the residue from broth cultures, washed agar growth, and whole unwashed agar cultures. Horder and Ferry concluded that the material contained in the washings of agar cultures, because it could be prepared in watery solution and was low in protein content, as well as being practically non-toxic and high in antigenic properties, more nearly approached the ideal antigen than any preparation which had come under their observation. Barach (1928)[74-5] reported that Berkefeld filtrates of pneumococci shaken in salt solution and also filtrates of broth cultures caused in mice the development of protection which appeared on the fourth day after injection. The immunity produced was dependent upon type-specific protective substances and not upon the elaboration of the common protein antibody.

Another method of preparing antigens from broth cultures of pneumococci was that described by Maeji (1930),[850, 853-4] who employed the technique of Besredka for preparing antivirus. The supernatant fluid from centrifuged beef-serum bouillon cultures of Type I Pneumococcus, when injected subcutaneously into rabbits, gave partial protection against infection with a Type I strain. In the case of Type II Pneumococcus the protective substance appeared only when goat serum was added to the medium, while with Type III organisms no antivirus could be obtained. Maeji claimed that after the administration of Type I antivirus by inhalation,

young rabbits resisted intratracheal inoculation with a virulent culture, and that resistance could be increased by repeated inhalation of the preparation. The attenuating action of filtered broth cultures of pneumococci on fresh cultures of the same organism was reported by Okischio (1932),[1023] but the use of anti-virus either for the preparation of vaccines or for the treatment of pneumococcal infections has not come into general use.

There are yet other references to the use of extracts and autolysates of Pneumococcus for the purpose of inducing immunity. Among them might be mentioned those of Day (1930 and 1934)[307, 309] who reported that the intraperitoneal injection into mice of autolysates of both Type I and Type II cultures was followed one week later by protection against one thousand to ten thousand fatal doses of culture. Another communication was that of Harley (1934)[591] in which was reported the preparation of antigens by extracting Type I and II pneumococci with 0.05N hydrochloric acid at 60°.

TISSUE EXTRACTS AND EXUDATES

The possibility that products arising from the vital activities of Pneumococcus in animal tissues might exert immunizing action and that such products might be applied to the protection or treatment of animals and human beings has not been neglected. Foà and Carbone,[463] Bonome (1891),[137] and Mosny (1892)[932] were thus able to render rabbits relatively invulnerable to the injection of otherwise lethal doses of pneumococcal cultures. Hartman (1913)[598] studied the antigenic action of the principal constituents of pneumonic exudates, but the results were largely negative. Freedlander (1928)[480] employed saline extracts of organs of infected rabbits as antigens and, after three injections at five to seven-day intervals into normal rabbits, obtained a serum of high protective titer for mice against Type I Pneumococcus and a serum of somewhat less potency against a Type II culture. While the serums compared favorably in protective action with thera-

peutic Type I antipneumococcic serum, they were low in agglu-
tinins, contained no precipitins, possessed no remarkable opsonic
powers, and displayed no direct bactericidal action.

The idea was revived in 1930 by Banzhaf and Curphey,[71] who
combined in horses intramuscular injections of phenolized pneu-
mococcal pleural exudates with intravenous injections of the for-
malinized sediment from broth cultures of pneumococci. When
tested by Goodner's method the therapeutic value of the serum was
disproportionate to the mouse-protective action, which fact Banz-
haf and Curphey interpreted as indicating the possible presence
of added therapeutic substances in the serum resulting from the
addition of exudate to the usual method of immunization.

Toxins and Hemotoxins

SO-CALLED TOXINS

The possible existence of toxins either in the pneumococcal cell
or resulting from its metabolic processes has already been dis-
cussed in Chapter III. It is extremely doubtful if Pneumococcus
possesses or produces a true toxin, but the idea still survives, and
the action of some therapeutic serums, quite apart from their
tropic, agglutinative, precipitative, or protective effects, in amel-
iorating the intoxication of pneumonia in man, has encouraged
the search for a toxic principle with the hope of producing an
antitoxin.

The Klemperers (1892)[725] were convinced that they had suc-
ceeded in isolating a toxic protein from Pneumococcus, which they
named "Pneumotoxin," but the action of the substance can now be
explained on other grounds. In 1897, Auld[30] separated an albu-
mose and an organic acid from local lesions, lungs, and spleens of
animals infected with Pneumococcus. No adequate proof could be
obtained that the protein was a true toxin, but Auld intimated that
a true toxin might in all probability be united with the protein.
The albumose preparations had a certain immunizing value, but no

evidence was advanced to show that the immunity was antitoxic in nature.

In 1912, Rosenow[1166] obtained from pneumococci a poisonous substance, similar to histamine in its action, but wholly incapable of provoking any immune response. Larson,[788] and then Olson (1926),[1029] believed that Pneumococcus contained a toxin and that by injecting animals with whole cultures of the organism treated with sodium ricinoleate, or with autolyzed broth cultures, it was possible to produce an antitoxic serum. The assumption was based solely on the physiological effect of the serum and not on any demonstration or titration of the antitoxin which the serum was supposed to contain.

The work of Parker[1061] and of Parker and McCoy (1929)[1062] is more suggestive. By allowing pneumococci of Types I, II, and III to autolyze under strict, anaerobic conditions, the authors obtained a preparation that was toxic for guinea pigs and for horses and which, when injected repeatedly into horses over a period of eight months, caused the animals so treated to develop in the serum substances capable of neutralizing *in vitro* the toxic autolysates. Parker reported that antipneumotoxic serum produced in rabbits or horses by immunization with sterile filtrates of pneumotoxin afforded heterologous protection to guinea pigs—at least as far as Type I or Type II pneumococci were concerned—against the development of pneumonia, while the serum of some of the treated horses exerted a curative action against infection with pneumococci of the homologous type, but contained little if any antitoxin for heterologous types. Furthermore, since the serums contained no specific protective antibodies against pneumococci, Parker concluded that the serum was antitoxic in action.

The experiments of Parker and McCoy, including a definite determination of the toxic power of sterile pneumococcal autolysates and of the neutralizing strength of serum produced in response to the injection of the autolysates, with evidence of the absence of specific protective antibodies, constitute the first accurately con-

trolled observations of the kind and are sufficiently suggestive to encourage further study of pneumococcal poisons and of their antigenic properties.

Another report of a similar nature was that coming from Jamieson and Powell (1931)[676] who obtained from pneumococci toxic substances not unlike those which have been demonstrated by other workers as being produced by various streptococci. The action of the substances could be demonstrated by skin tests on human beings and on certain breeds of rabbits. By the same method, the serum of horses treated by subcutaneous injections of the toxic substances could be shown to possess neutralizing properties for the alleged toxin. The neutralizing principle could be concentrated to a moderate degree in the refinement of globulins by the usual chemical procedures and, according to Jamieson and Powell, was independent of the small amount of protective antibodies present in the serum.

HEMOTOXINS

In 1927, Neill, Fleming, and Gaspari[958] described the results of studies on the antigenic or immunizing action of various preparations of pneumococcal hemotoxin. The power of the reduced and of the reversibly oxidized forms of hemotoxin to evoke immunity were identical, but preparations in which the hemolytic principle had been rendered irreversible or destroyed by heat or by high concentrations of hydrogen peroxide were inactive in this respect. The serum of rabbits and of horses injected with active hemotoxin neutralized the substance, while the antigenic action of the hemotoxin appeared to be independent of the protein fraction of the cell.

Methods of Administering Antigens

The manner in which Pneumococcus or its several antigens are introduced into the body of an animal of any given species, other factors being constant, may determine both qualitatively and quantitatively the nature of the immunological response. In the early

studies on Pneumococcus one finds references to the subcutaneous
injection of the organism or its products. The introduction of the
intravenous route was followed by the more rapid appearance and
a higher level of immunity in the treated animals. The administra-
tion of antigens by way of the peritoneal cavity also offered ad-
vantages over the subcutaneous route both in time of development
of immunity and in heightened resistance.

In 1922, Bronfenbrenner and Knights[149] published the results of
a study on the comparative efficacy of the subcutaneous, intrave-
nous, intratracheal, and intrapleural routes for the introduction
of pneumococcal antigens. Rabbits were used and the immunity
evoked was measured by the bactericidal, opsonizing, and protec-
tive power of the serum following the different methods of treat-
ment. The bactericidal titer of the serum from rabbits receiving
intravenous and subcutaneous injections, as determined by the
Heist and Solis-Cohen[634] technique, was approximately the same
and was greater than that of the serum of animals injected either
by the intrapleural or intratracheal routes. Both the intratra-
cheal and intrapleural methods induced a much higher opsonic
content of the immune serum than did the other procedures, while
the administration of antigen by way of the blood, or into the tra-
chea or pleura, caused the rabbits to yield serum containing pro-
tective antibody, whereas the serum from rabbits treated subcu-
taneously failed to confer passive protection on mice.

In communications published in 1924, 1927, and 1930, Still-
man[1330-2, 1334-5] supplied additional information concerning the
nature and degree of the immune response in mice and rabbits re-
sulting from inhalation, and from the subcutaneous, intramuscu-
lar, and intravenous application of both living and dead pneumo-
cocci. The serum of 80 per cent of rabbits injected intravenously
with fixed amounts of heat-killed pneumococci and the serum of 60
per cent of the animals injected intraperitoneally with similar
doses contained agglutinins and all showed protective antibodies.
Of rabbits receiving intramuscular injections, the serum of only 33

per cent contained agglutinins although 86 per cent were protective. In the case of animals receiving antigen by the subcutaneous route, none produced agglutinins although protective substances could be demonstrated in 71 per cent of the individual serums.

With skin sensitivity and the presence of type-specific agglutinins and protective substances in serum as criteria for the strength and specificity of pneumococcal antigens, Harley (1935)[592] obtained a higher degree of immunity and far greater type-specificity when vaccines of smooth and rough organisms were introduced intravenously than when injected intradermally.

Killian (1924),[706] by continued subcutaneous and intraperitoneal injections—the so-called bigeminal method—obtained a higher degree of protection in mice than by the subcutaneous route alone, and he believed that the fact argued against the acceptance of the hypothesis that a purely local immunity was necessary for establishing a state of protection against pneumococcal infection. By intravenous injection, extremely small quantities of antigen sufficed to produce protection, the immune state being demonstrable in an increasing degree from the third day after injection. Killian reported that a short refractory period ensued after the administration of antigen, and accordingly, delayed further antigenic stimulation for four days until the period had passed.

It is reasonable to assume that the duration of the refractory period—or the negative phase, as Wright termed it—depends on the route employed in the injection of antigen, the ability of the body cells to respond to subsequent stimulation being contingent upon the rate of absorption of antigenic material from the site of the injection. From foci under the skin, absorption would be less rapid than that from the peritoneal cavity and, quite obviously, the utilization of antigen would be most rapid when the injection is made directly into the blood stream. Because of the slower seepage of antigenic materials from the subcutaneous tissues, that route is preferred for the production of antitoxin, but for the

elaboration of antibacterial substances, the intravenous method is the one of choice.

INTRADERMAL INJECTION

The intradermal injection of pneumococci and their products may be followed by the development of the immune state. Goodner (1928),[525-6] in studying the pathogenic action of living pneumococci inoculated into the skin of rabbits, observed that the serum of animals surviving infection had acquired protective antibodies. Furthermore, within five days after a single intradermal injection of dead pneumococci, the normal rabbit developed resistance to infection. In two communications appearing in 1930, Julianelle[687-8] reported that repeated injections of small doses of suspensions of heat-killed pneumococci of Types I and III into the skin of rabbits induced species-specific but in no case type-specific antibodies for the organisms. However, animals developing only species-specific antibodies after intradermal vaccination still possessed the ability to form type-specific immune substances when subsequently given intravenous injections of pneumococci of fixed types. Despite the failure of heat-killed pneumococci when injected intradermally to evoke the formation of type-specific antibodies in the rabbit, devitalized cultures do, nevertheless, render the animal actively immune to infection with pneumococci not only of homologous but also of heterologous type. The injection into the skin of soluble derivatives of Pneumococcus in Julianelle's experiments was not followed by the production of active immunity. The destruction or inactivation in the skin of rabbits of type-specific antigen injected intradermally was later observed by Harley (1935).[593] The somatic protein, on the contrary, appeared to be unaffected and stimulated the formation of species-specific antibodies.

The observation of Julianelle was significant in that it revealed a hitherto unknown property of the epidermal cells of the rabbit.

The ability of that animal when injected intradermally to elaborate species-specific antibody, taken with the inability to manufacture type-specific antibody when similarly injected, points to a partial disruption of the molecular configuration of the pneumococcal antigen during its sojourn in the skin. The type-specific fraction of the antigen is probably destroyed and there remains only the somatic protein and carbohydrate of the bacterial cell to stimulate the immunological mechanism. That the mechanism is, nevertheless, fully capable of functioning in producing type-specific immune substances is shown by the response of the rabbit when the antigen is introduced directly into the blood stream. The hypothesis is further supported by Julianelle's observation that the intradermal injection of heat-killed R forms as well as S forms of pneumococci calls forth the production of active immunity. Julianelle therefore demonstrated that the route of administration of pneumococcal antigen, at least in the rabbit, may be a determining factor in the qualitative nature of the immune response.

An analogous process taking place in the human body was reported in the same year by Francis and Tillett.[478] The repeated injection of the specific capsular polysaccharide of pneumococci of Types I, II, and III into the skin of patients suffering from lobar pneumonia was followed in the second or third week of convalescence by the appearance of circulating antibodies for one or more heterologous types. However, in none of the normal controls was the phenomenon observed. In the next year (1931), Finland and Sutliff[446-7] confirmed the observations of Francis and Tillett. Pneumonia patients receiving repeated intracutaneous injections of soluble specific substance from Type I, II, and III pneumococci, in a week or more developed in the blood agglutinins and protective antibodies sometimes heterologous for the infecting type of organism but, unlike the effect in the rabbit, the immune bodies were homologous for the type of capsular carbohydrate injected.

In a subsequent report (1932), Finland and Sutliff[448] announced that the simultaneous intradermal injection into normal human be-

ings of specific polysaccharide of the first three pneumococcal types and of protein and autolysates derived from organisms of Types I and II was followed by the appearance or increase of the pneumococcidal power of whole defibrinated blood and in most instances by the presence of agglutinins and protective antibodies for one or more types of pneumococci. A single intradermal injection of 0.01 milligram of the capsular polysaccharide of pneumococci of Types I, II, or III, or four similar daily injections, resulted in the development of antibodies corresponding in type only to that of the antigen injected. A single injection into the skin of autolysates obtained from virulent Type I, II, and III pneumococci gave rise to an increase in the pneumococcidal power of whole blood and the appearance of homologous type-agglutinins and protective antibodies in about one-third of the normal subjects. A similar injection of pneumococcal protein, on the contrary, failed to evoke specific antibodies to any appreciable degree.

Further evidence of the antigenic action of specific polysaccharides injected into the skin of normal individuals was presented by Zozaya and Clark[1590] who, after the intradermal injection of soluble specific substance of pneumococci of Types I, II, and III were able to demonstrate homologous precipitins and protective antibodies in the serum of human beings, who gave no history of recent pneumococcal infection. Another observation of these authors may be repeated here. It was found that by increasing the surface area of the carbohydrate by adsorption on charcoal or celloidin particles it was possible to evoke antigenic properties in preparations that otherwise appeared to lack immunizing action and to enhance the action of preparations that already possessed these properties. Francis,[474] in a still more recent paper (1934), described experiments in which he studied the antigenic action of the original and the acetylated forms of the specific polysaccharide of Pneumococcus. Weekly doses of 0.01 milligram were administered intradermally over a period of three weeks to normal individuals. The experiment showed that both the original soluble specific sub-

stance and the acetylated polysaccharide of Pneumococcus stimu-
lated the development of type-specific agglutinins, precipitins, and
mouse-protective antibodies in the serum of normal human beings.

A consideration of the work of Julianelle, of Francis and Til-
lett, of Finland and Sutliff, and of Zozaya and Clark demonstrates
that the antigenic constituents of Pneumococcus introduced by the
intradermal route may act as immunizing agents and that, while
in man both type-specific immunity and corresponding type-spe-
cific antibodies may be developed, the skin of the rabbit possesses
the power so to alter pneumococcal antigen that only species-spe-
cific immunity results. The difference in the epidermal and possibly
the other somatic cells of animals of diverse species in their reac-
tion to the parenteral introduction of Pneumococcus and its de-
rivatives offers a field for study which should aid in clarifying some
of the questions concerning the physiological processes involved in
the development of the immune state.

INHALATION AND INTRABRONCHIAL INSUFFLATION

After giving repeated inhalations of Type I Pneumococcus,
Stillman[1331-2] demonstrated agglutinins and protective antibodies
in the serum of rabbits so treated. Agglutinin production appeared
to remain stationary after the fifth exposure to antigen, while the
protective antibody content steadily rose. Repeated inhalations of
killed pneumococci by mice resulted in only a slight degree of im-
munity, but when living organisms were similarly administered a
definite degree of active immunity could be induced in the animals.

Eguchi (1925)[351] confirmed Stillman's observation on the pro-
tection developing in mice following the inhalation of killed pneu-
mococci, and found further that immunity could be induced by
organisms of Type II as well as those of Type I. Maeji[853] also re-
ported that, in the case of antivirus, inhalation was an effective
mode of protecting young rabbits against subsequent infection
with Pneumococcus, and that the degree of protection increased

with the quantity of inhaled antivirus and the number of applications.

Although Cooper (1926)[276] failed in attempts to immunize rabbits to Pneumococcus by either the subcutaneous or intradermal injection of heat-killed cultures, he was able by applying the vaccine beneath the buccal membrane of the cheek to protect the majority of rabbits so treated for a period of four months against a fatal dose of the organism. Instillation of the vaccine into the eye or swabbing the nose and mouth with the agent failed to result in any effective immunity. Stuppy and Falk (1928)[1351] reinvestigated the subject presented by Cooper. Rabbits receiving ten to twenty daily injections of heat-killed Type I pneumococci into the buccal mucosa later survived inoculation with 1,000 M.L.D. of the same strain; of the animals receiving five preliminary injections three out of four lived; while all rabbits given only one or two injections died. Ten daily subcutaneous, intradermal, intravenous, and intratracheal injections or the administration of the vaccine by intratracheal insufflation or by intraocular instillation (contrary to the claims of Cooper) protected the animals against subsequent infection, although vaccination by insufflation or instillation was less reliable as an immunizing procedure than was the administration of the killed culture into the buccal submucosa or into the trachea.

Stuppy, Cannon, and Falk[1350] rendered rabbits immune to intraperitoneal infection with pneumococci of Types I and II by the daily insufflation into the nose and throat of suspensions of heat-killed cultures of homologous type, but failed in similar attempts with Type III organisms. In rabbits so treated, the insufflation of living, virulent pneumococci induced a proliferative and exudative type of reaction in the lung in which the macrophage was the predominant cell, accompanied by considerable numbers of eosinophiles. This altered reactivity of the pulmonary tissues was interpreted by the authors as affording evidence of a definite, localized

type of tissue immunity in the lung. Heterologous cultures of liv-
ing pneumococci induced the local immune reaction but to a lesser
degree.

ORAL ADMINISTRATION

When pneumococci enter the body by way of the alimentary
canal, their constituents may eventually reach the body cells re-
sponsible for the creation of immune bodies. In 1925, Eguchi[351] re-
ported that attempts to immunize mice by feeding them Type I
pneumococci met with some success when young mice were used but
with none in the case of adult mice. Kimura (1927),[710] neverthe-
less, was able to protect a large percentage of adult mice against
one hundred to one thousand lethal doses of Type I pneumococci
by dropping into the mouths of the animals large amounts of heat-
killed cocci of the corresponding type. A similar result attended
Maeji's attempts to induce active immunity in mice by spraying
the mouth with suspensions of pneumococci of Types I and II.
When the effort was successful, there developed cross-protection,
especially between Types I and II, with some protective action
against Type III strains. Maeji[849] subsequently (1929) was able
to immunize young mice, rats, and rabbits by the daily feeding of
large quantities of broth cultures of highly virulent Type III or-
ganisms. Although young rabbits could be rendered resistant to in-
fection with Type III pneumococci by the oral administration of
cultures of the corresponding type, no agglutinins could be de-
tected in the serum of the animals.

For the purpose of increasing the permeability of the intestinal
mucosa for pneumococcal antigen, McDaniels (1931)[876] fed young
white rats first with egg-white and then thirty minutes later with
autolysates of Type I Pneumococcus. When tested after the lapse
of five days by intraperitoneal inoculation with a virulent strain
of Type I Pneumococcus, all the animals showed toleration of
many lethal doses of culture, while the rats that had received a
preliminary feeding with egg-white exhibited a greater resistance

to infection than did those that had not been so fed. In a second communication (1932), McDaniels[877] reported attempts to ascertain the details of the immunological mechanism operating in orally immunized rats. Normal control rats and rats previously injected with egg-white and Type I pneumococcal autolysates were inoculated intraperitoneally with one hundred fatal doses of a virulent, homologous strain of Pneumococcus. All the normal rats died within two to four days, while 96 per cent of the vaccinated animals survived. Although the leucocyte count was subject to wide fluctuations, the immunized rats, six hours after inoculation, always showed an appreciably higher white-cell count than did the unvaccinated controls. In none of the vaccinated rats which survived infection could pneumococci be demonstrated in the blood, although pneumococcemia was present in all the animals that succumbed.

Ross (1931)[1188] described the comparative immunizing action of soluble specific substance and of whole or dissolved pneumococci when fed to rats. One feeding of Type I SSS sufficed to protect the test animals against intraperitoneal injection of virulent Type I organisms, and the increased type-specific resistance was comparable, with slight quantitative differences, to that following the oral administration of intact or dissolved pneumococci. Ross failed to immunize mice by a similar procedure and attributed the failure to some cell constituent active in the mouth of mice. In other papers (1932), Ross[1189-90] reported that the capsular polysaccharide of Type II Pneumococcus was incapable, when introduced by the oral route, of increasing the resistance of rats to infection. The soluble specific substance of Type III organisms induced protection in rats to organisms of the homologous type but the percentage of animals so protected was less than in the case of animals receiving the whole or intact cell by mouth. When the specific polysaccharide of Type I Pneumococcus was fed to rats, no immunity against Type II or Type III organisms was obtained, and the ingestion of SSS of Types II and III did not protect the

animals against Type I organisms. An observation of Ross on the
fate of the ingested polysaccharide was that a very large propor-
tion of the substance, when fed to rats, appeared in the feces.

Further experiments on oral immunization against Pneumococ-
cus, when applied to rabbits, were described in 1932 by Kolmer and
Rule.[745] The antigens comprised a) pneumococci of Types I, II,
and III cultivated in sterile milk for twenty-four hours and heated
at 60° for one hour, b) twenty-four-hour broth cultures of the
same types exposed for two hours to a N/15 concentration of hy-
drochloric acid, and c) sedimented cocci from twenty-four-hour
broth cultures subsequently treated with sodium taurocholate. The
materials were administered daily for seven days through a stom-
ach tube, and one week after the last treatment the rabbits were
inoculated intratracheally with living broth cultures. While to
Kolmer and Rule the acid-killed cocci, of the three kinds of prepa-
rations used, appeared to engender the highest degree of immu-
nity, the figures are not significant because of the lack of uni-
formity of the results and the low virulence of the Type II and
Type III cultures employed in testing the resistance of the rabbits.

In a second report (1933), Kolmer and Rule[747] stated that the
oral administration of acid-killed vaccines made from Type I, II,
and III pneumococci, in doses representing 100 to 1,000 million or-
ganisms per kilo of body weight, were effective in rendering rabbits
immune to subsequent intratracheal infection with pneumococci of
the homologous type. Although occasionally a single dose of 1,000
million cocci produced active immunity against infection by Type
I Pneumococcus, the best results were obtained with a minimum of
five daily doses. The immunity thus induced was transient, de-
creasing after one month's time and practically disappearing in
six months. Vaccines introduced by way of the mouth were inferior
in immunizing action to similar agents injected subcutaneously.
Using monkeys as test animals, and employing vaccines of pneumo-
cocci killed by tricresol and by hydrochloric acid, Kolmer and

Rule confirmed the superiority of the subcutaneous over the oral route of administration.

Host Response

As in all physiological processes there are periods in the development of an animal when the somatic cells function more actively in absorbing alien material and in creating and extruding immune substances into the circulating blood. This power may be lacking in very young animals, but it appears to increase as the animal grows and reaches a stage of full vigor, and then to decline as the metabolism of the body cells wanes. In addition to quantitative differences due to age in the antibody content of serum of rabbits immunized against Pneumococcus, Baumgartner (1934)[91-2] detected qualitative variations as well in the antibodies elaborated by young and older animals. The fact is sufficiently well established in immunological practice to require no detailed discussion.

Antagonistic Action of Soluble Specific Substance

In preceding chapters, mention was made of the aggressin-like action of soluble specific substance, of the zonal effect observed when this substance was used as an immunizing agent, and of its antagonistic behavior in reactions between immune serum and Pneumococcus or its derivatives. The soluble specific substance, although non-toxic in itself, can enhance the invasive power of pneumococci (Felton and Bailey[419]). The phenomenon may be an additive effect which, in turn, may be due to some interference with the natural defense mechanism of the body. Whatever the explanation of this property of specific polysaccharides, it is a fact to be reckoned with in the production of active immunity, in testing the immunity established by the administration of pneumococcal antigens, and in measuring the potency of antipneumococcic serum.

Felton and Bailey demonstrated that the immunizing action of soluble specific substance operated within certain prescribed lim-

its. A definite minimum of the material was obviously necessary to incite the formation of antibodies, but when the dose was increased, a point was reached beyond which no immunity developed. Saito and Ulrich (1928)[1214] also observed this zone of antigenic effectiveness with a carbohydrate preparation in producing a state of protection in animals. A similar observation on the cellular carbohydrate was reported by Wadsworth and Brown (1931).[1466-7] There can be no doubt that pneumococcal antigen, particularly the capsular polysaccharide, in excess of a certain optimal amount hinders its own immunizing action.

Another manifestation of the antagonistic action of the soluble specific substance is seen in the blocking of the reaction between pneumococcal antigen and homologous antibody. Felton and Bailey[419] drew attention to the inhibiting power of SSS frequently noted in precipitin and protection tests when amounts of antigen and serum greater than the optimum are used. The authors ascribed the antagonism to the presence of precipitable residues in immune serum as well as to an excess of soluble specific substance in the antigen. The work of Felton and Bailey and that of Sickles (1927)[1277] point to at least one factor responsible for the inhibiting power of the soluble carbohydrate. Through its precipitating action, the polysaccharide removes some of the protective antibody from immune serum and at the same time (Sickles) interferes with phagocytosis, consequently robbing the serum of protective properties. The observation recalls that of Sia (1927),[1268] who found that Pneumococcus could adsorb normal opsonin from the serum of animals naturally resistant to pneumococcal infection—an observation later confirmed by Ward (1930)[1480-1] for normal human whole blood, and who also, like Sia, noted that the action was type-specific. Ward suggested that the zone of inhibition was caused by the specific precipitate formed by the antiserum and homologous capsular polysaccharide, which interfered, perhaps mechanically, with the ingestion of the pneumococci by leucocytes.

Enders and Wu (1934)[362] reported that the A carbohydrate

from Pneumococcus possessed a greater anti-opsonic action than the deacetylated polysaccharide. The opsonic titer of normal human serum was practically nullified by the addition of the A carbohydrate, while in immune serum the A carbohydrate brought about a greater diminution in opsonic activity than did its derivatives. The result was therefore a complete inhibition of the pneumococcidal action of normal serum and a partial blocking of the same action of specific immune serum. The inhibitory or antagonistic behavior of the carbohydrate fraction of Pneumococcus calls for careful discrimination in the selection of the antigenic dosage of. pneumococcal materials for the production of active immunity and also for conducting serological reactions which have to do with the immune state.

Summary

The substance of the material presented in this chapter may be expressed as follows: The immunizing properties and type-specific antigenic action of Pneumococcus and its derivatives are directly proportional to the virulence of the culture employed and appear to be the same whether the organism is in a living condition or appropriately killed by heat or by formalin, the integrity of the immunizing principle being better preserved in suspensions of heat-killed than of formalinized pneumococci. Filtrates from fluid cultures and watery or saline extracts of pneumococci, representing as they do only a part of the antigenic components of the pneumococcal cell, are more limited in immunizing properties than the entire cell. Although specially prepared broth cultures may exhibit a toxic action in animals, it does not necessarily follow that the injection of the alleged toxins results in the production of pneumococcal antitoxin. To be sure, some evidence of such a possibility has been presented but at the present time it would be going beyond the facts to say that Pneumococcus contains or elaborates a true toxin or that antitoxic processes are a recognized part of the immunological mechanism against Pneumococcus.

Because of simplicity of preparation, stability, and freedom from undesirable secondary action, heat-killed vaccines are preferable to those prepared with bile, bile salts, soaps, or other solvents or devitalizing agents. Sensitized cocci present no advantages in immunizing properties over cells untreated with specific immune serum.

The route by which antigens are introduced into the animal body affects both the kind and quantity of specific antibodies called forth. Injection into the venous system is the most effective method for inducing a high degree of immunity and strict type-specificity of the antibodies. Injection into the peritoneal cavity, into the muscles, or under the skin likewise leads to the elaboration of antibodies but, owing to the respectively slower absorption of antigen, there is a broadening of the specificity of the immunological response. Injection of pneumococcal substance into the skin stimulates the formation of antibodies but type-specificity may be sacrificed for species-specificity. The introduction of antigen into the bronchi by inhalation, insufflation, or injection, and also by enteric paths, is even less effective in raising the level of resistance or in evoking demonstrable immune substances in the circulating blood.

The age of the animal treated with immunizing agents is another condition that determines the ability of animals to respond, at least in a quantitative way, to antigenic stimuli, while different species of animals display various physiological traits when injected parenterally with Pneumococcus or its derivatives.

The antagonistic effect of excessive doses of pneumococcal material, particularly of the specific capsular polysaccharide, may again be emphasized, since the amount of the soluble specific substance in a vaccine influences the development of the immune state or of immune substances.

These, then, are the several factors which condition the immunological behavior of animals toward substances of pneumococcal origin and which qualify any definition of the term antigen.

CHAPTER XI

ANTIBODIES TO PNEUMOCOCCUS

*The nature of the immune substances appearing after the injec-
tion of Pneumococcus, its constituents, or products into the animal
body; the detection and estimation of specific antibodies in im-
mune serum; with a brief discussion of the factors which operate
in establishing immunity to Pneumococcus.*

THE introduction into animals of pneumococci, their deriva-
tives, or their several metabolic products generates a variety
of antagonistic substances which may serve to protect the animal
against subsequent infection with virulent members of this bac-
terial species. In the present chapter there will be discussed the
substances to be found in the circulating blood after active immu-
nization and analogous substances normally present in animals, as
well as changes in the somatic cells contributing to the immune
state induced by parenteral injection of pneumococcal materials.

The manifestations of the physiological functions aroused by
the administration to animals of different species of pneumococci
in one form or another are many and varied. Furthermore, the
substances evolved in the immune processes are so closely related
and so intimately mingled in immune serum that a discussion of
any one of the specific substances necessarily involves that of other
associated antibodies. However, in reviewing the investigations of
earlier workers, one finds that the various immunological effects
were discovered one by one, until the mosaic of pneumococcal im-
munity—still incomplete, to be sure—has assumed a definite pat-
tern which can now be recognized by improved serological and
biochemical methods.

Agglutinins

The first tangible effect referable to an altered bodily state fol-
lowing the injection of pneumococci was that of agglutinin for-

mation. Meager as was Metchnikoff's[894] description of the phenomenon, it was undoubtedly he who, in 1891, first observed the clumping of pneumococci in immune serum. Issaeff,[673] two years later, confirmed Metchnikoff's observation, and added that the serum of rabbits vaccinated with Pneumococcus when mixed with the organism, instead of becoming cloudy, developed a deposit. It is probable that in one of Mosny's[933] experiments (1892) agglutination took place although he interpreted the result in a different way. The incubation of a culture of Pneumococcus in normal rabbit serum was attended by a diffuse clouding of the medium with a granular sediment appearing after twenty-four hours, while in immune serum the faint cloud disappeared after eight hours due very likely to the sedimentation of agglutinated cells. In 1896, Washbourn[1486] undoubtedly observed agglutination, for he described the sediment accumulating in immune rabbit serum twenty-four hours after the addition of pneumococci as consisting "of pneumococci staining well and grouped in masses."

It was Bezancon and Griffon[108-10] who, in 1897, recognized the phenomenon as agglutination and compared the effect in the case of Pneumococcus to the Widal test for the diagnosis of typhoid infection. Although there was no difficulty in demonstrating the agglutinative properties of fluids collected from animals dying from pneumococcal infections, the results obtained with serum from patients suffering from similar infections were somewhat confusing. However, Bezançon and Griffon gave a correct interpretation to their results in concluding that from the standpoint of agglutination there existed several races of pneumococci. In the first five cases of pneumonia studied, the organisms isolated agglutinated well in immune serum and these strains were referred to by the authors as *vulgaire* pneumococci. By the agglutination method, the French workers differentiated a strain with all the characteristics of Type III Pneumococcus and were therefore the first bacteriologists to recognize the applicability of the agglutination reaction to the differentiation of Pneumococcus from re-

lated bacterial species and to the serological classification of pneu-
mococci.

In 1902, Neufeld[974] took exception to some of his predecessors
who had studied agglutination of pneumococci with specific serum.
He excluded any experiments in which only thread-like growths
were observed, and contended that in none of the previous studies
was the serum diluted, nor were the observations made soon enough
after the organisms and immune serum were mixed. It was in this
communication that Neufeld first described the *Quellung* effect
which appeared when homologous immune serum was added to
pneumococci. Neither normal rabbit nor normal human serum
agglutinated pneumococci but normal beef serum often caused
clumping of the organisms. Neufeld also noted that dead as well as
living pneumococci were susceptible to the clumping action of im-
mune serum, and that in the reaction the cocci were not dissolved
nor were the living organisms killed. Neufeld met with difficulties in
preserving the agglutinative titer of some samples of immune rab-
bit serum—a difficulty later experienced by Hintze (1921)[647] and
others with rabbit serum. More recently, Valentine, McGuire,
Whitney, and Falk (1931)[1443] reported that the agglutination
titer of dried antipneumococcic serum kept at room temperature
decreased more rapidly than the mouse-protective titer. However,
when the desiccated serum preparations were preserved at ice-box
temperatures, the potency of the serum in this respect was not ap-
preciably affected.

AGGLUTININS IN THE BLOOD OF PNEUMONIA PATIENTS

The serum of patients convalescing from pneumonia was in some
cases found by Neufeld to contain agglutinins. Sometimes their ap-
pearance was observed shortly before crisis although the aggluti-
nating power of convalescent serum was highest on the day after
crisis. The later observation was duplicated in the same year by
Huber,[663] who also reported that the agglutinative properties of
convalescent serum progressively diminished and completely dis-

appeared ten days after crisis. Similar results were obtained by Rosenow (1903),[1158] who found that when culture and serum came from the same patient agglutination was most pronounced, but in none of the serums from sixty-five cases of pneumonia when tested against twenty-five strains of Pneumococcus did agglutination fail to take place. Jehle (1903)[678] was able to demonstrate relatively high agglutinating power in the serum of all cases tested of croupous pneumonia terminating in crisis but, contrary to Huber and Rosenow, Jehle observed that only comparatively small amounts of agglutinin were demonstrable forty-eight hours after crisis and that the antibody had practically disappeared four days later.

In a second communication, Rosenow (1904)[1159] reported additional tests on the serum of pneumonia patients which confirmed his earlier results, and made the observation that pneumococci replanted from the agglutinated mass in immune serum were viable as long as thirty days after being agglutinated. Wadsworth[1455] substituted for the whole culture as antigen saline suspensions of pneumococci sedimented from twenty-four to thirty-six-hour-old broth cultures and carried out agglutination experiments with immune serum obtained from rabbits which had previously been injected with dead and later with living pneumococci. With serum from pneumonia patients in dilutions of 1 to 10 to 1 to 20 the antigen gave positive reactions in five or six hours. Wadsworth confirmed Neufeld's negative results for normal rabbit and bullock serum, but found that normal human serum in a dilution of 1 to 10 agglutinated pneumococci in less than eighteen hours. When the serum was diluted in a proportion of 1 to 30, no agglutination took place. In 1905, Longcope[824] described the sediment occurring when pneumococci were added to serum obtained from pneumonia patients, and also the accompanying swelling of the bacterial capsule, previously observed by Neufeld.

In a study of the various characters of a large number of strains of pneumococci from a variety of human sources, Kindborg (1905)[713] obtained immune rabbit and sheep serum of high agglu-

tinin titer, but concluded that agglutination was specific only for the strain used in the preparation of the serum employed in the test. In the same year, Collins,[270] after immunizing rabbits successively with heat-killed and then with living broth cultures, tested the serum so prepared against some seventy strains of Pneumococcus. The serum of an animal immunized with one particular strain of Pneumococcus agglutinated only seven of the organisms when used in a dilution equalling that in which the homologous organism agglutinated.

Collins concluded from her study that pneumococci by reason of their agglutinative properties exhibit a tendency to separate into numerous groups, and also that by the reaction of agglutination *Pneumococcus mucosus* forms a distinct and consistent variety. The conclusions were based on results obtained by the method of agglutinin-absorption, which indicated that the agglutinating substances consist of specific and group agglutinins. Collins, appreciating that pneumococci showed marked differences in ability to undergo agglutination, and believing that there existed different types or groups as far as their agglutinative ability was concerned, was convinced that it was not possible to establish a definite relationship between the agglutination reaction and other characters of pneumococci except in the case of *Pneumococcus mucosus*.

Two other reports published before the serological classification of pneumococci was established are those of Panichi (1907),[1047-8] and of Cotoni and Truche (1912).[284] The former author, after testing the agglutinative property of a strain of Pneumococcus at different stages of growth in bouillon when added to serum from rabbits, sheep, and asses previously injected with the same culture, concluded that agglutinability of the organism was greater during the process of growth than after the cocci had attained full development. Curative serums, including one prepared by Pane, did not necessarily contain agglutinins. The experimental data suggest that the serums employed were of low potency and, hence, the

results are not significant. Cotoni and Truche tested some thirty-one miscellaneous strains of pneumococci isolated from men, guinea pigs, rabbits, and horses against thirty-eight samples of serum from patients suffering from pneumococcal infections and against immune serum from horses and sheep, as well as normal serum, but the results of the agglutination reactions were confusing. For example, normal horse serum, under the conditions of the experiment, agglutinated the majority of the strains while normal sheep serum affected only a few of them. The action of the immune serums was indefinite, and Cotoni and Truche concluded that identification of pneumococci on the basis of agglutinability was uncertain.

The appearance of agglutinins in the blood during the course of lobar pneumonia was studied by Chickering (1914),[222] who found the antibody to be present in a large percentage of cases due to Groups I, II, and IV. In the most severe cases and in fatal cases, agglutinins could not be demonstrated nor could any be found, by the technique employed, in the blood of patients suffering from infection with Type III Pneumococcus. When agglutinins were demonstrable they usually appeared at the time of crisis, whereas in some cases agglutinins were present for only one day, while in others they persisted for several weeks. In infections due to pneumococci of Types I and II the reaction was always specific for the type of organism causing the infection, while in infections due to organisms of Group IV the strain was agglutinated only by a serum strictly homologous for that strain.

In an investigation of the antigen-antibody balance in lobar pneumonia, Blake (1918)[125] noted that prior to or coincident with the appearance of agglutinins, pneumococci disappeared from the blood. Patients who developed an excess of agglutinins over antigen invariably recovered; those who showed a progressive increase in the excess of antigen without the development of demonstrable antibodies invariably died. Clough[238] confirmed Chickering's findings in demonstrating pneumococcal agglutinins in approximately

FR. NEUFELD

three-fourths of the cases of lobar pneumonia tested, and sub-
stantiated Blake's observation that pneumonia patients who failed
to develop demonstrable agglutinins succumbed to the infection.

The agglutination curve of the blood of pneumonia patients re-
ceiving antipneumococcic serum was employed by Cole (1917)[258]
to explain variations in the curative action of the serum.

AGGLUTINATION AND SEROLOGICAL CLASSIFICATION

In studies carried on by Neufeld and Haendel[991] during the
years 1909 to 1912, it was learnt that agglutination of typical
and atypical pneumococci corresponded to susceptibility to the
protective action of specific immune serum. In 1913, Dochez and
Gillespie,[322] by the methods of mouse protection and agglutina-
tion, were able to separate strains of pneumococci isolated from
cases of lobar pneumonia into four groups. The close agreement in
the results demonstrated the value of the agglutination reaction
in differentiating pneumococci, while the absence of cross-agglu-
tination between the majority of representatives of the fourth
group attested the heterogenicity of that class. Hanes[588] applied
the method to cultures of Pneumococcus obtained from lobar pneu-
monia patients and, while confirming the work of Dochez and Gil-
lespie, encountered difficulties in the serological identification of
Type III organisms. In attempts to demonstrate specific agglu-
tinins in the serum of rabbits highly immunized to cultures of the
mucoid variety of Pneumococcus, the results were uniformly nega-
tive, but when the decapsulating method of Porges was used, in
every instance the organisms agglutinated with homologous Type
III serum, showed constant cross-agglutination within the group,
and failed to react with immune serum for Types I and II and
for streptococci. Hanes, therefore, suggested that henceforth the
name *Pneumococcus mucosus* should be adopted for organisms of
this group instead of the older designation, *Streptococcus mu-
cosus*. With the aid of the Porges technique, Nicolle, Jouan, and
Debains[1011] obtained agglutination with strains of Pneumococcus

that previously had failed to agglutinate with immune serum of the authors' preparation or with samples of serum from America.

Gillespie (1914)[516] reported that strains of Type I and II pneumococci exhibited narrow zones of agglutination whereas organisms of the other types showed broad zones, and ascribed the variations to the differences in the hydrogen ion concentration of the cultures used and to the greater susceptibility of pneumococci of Types I and II to the inhibiting action of salts. Armstrong (1921–1922)[19-20] placed greater reliance on the method of agglutinin-absorption than on simple agglutination in the classification of strains of pneumococci, since he believed that the latter technique was not always sufficient for recognition of type. On the basis of agglutinability, Olmstead,[1027-8] Griffith,[558] and later Cooper and her associates[272-4] extended the serological classification of pneumococci to include the present thirty-two separate and specific types.

AGGLUTINABILITY OF PNEUMOCOCCAL VARIANTS

The experiments of Stryker[1348] in 1916 demonstrated that the biological changes produced in pneumococci by growth in homologous immune serum included alterations in agglutinative behavior. In 1923, Blake and Trask[129] found that a similar treatment of pneumococcal cultures resulted, along with loss of virulence, in constant and distinct changes in agglutinability, with respect to both the character of agglutination and the zone of optimal reaction. The changes appeared to be not a gradual alteration of all members of a culture, but to consist in a rapid and complete change in individual organisms. Three main variants developed after growth in immune serum, and all exhibited marked differences in agglutinability. Analogous changes in the agglutinability of typical pneumococci after cultivation under unfavorable conditions were reported in the same year by Yoshioka.[1564] Cultivation on unsuitable media at 39° and long drying in the desiccator

brought about a marked decrease in the agglutinative ability of the cultures in the presence of homologous serum, and caused the development of agglutinability with heterologous serum. The changes appeared irregularly and suddenly. Megrail and Ecker,[888] on the contrary, claimed that Type I Pneumococcus in contact with Type I serum resisted the immunological change, as did the same organism when passed through a series of sterile abscesses artificially produced in mice and rats.

As has already been explained in Chapter V, the dissociation of typical pneumococci into degraded variants is accompanied by a loss of type-specificity and the development of species-specificity in the agglutinative properties of the organism. When the degenerative process has not extended too far and the organism is then appropriately stimulated by animal passage with the accompaniment of vaccines of typical strains or by growth in antirough serum, the organism may develop type-specific agglutinogens as it approaches the parent strain in its biological characters.

AGGLUTININS IN THE BLOOD OF ANIMALS

Bull (1915–1916)[170, 172-4] ascribed to agglutinins a decisive part in the resistance of animals to pneumococcal infection. He found that the injection of small quantities of specific antipneumococcic serum brought about almost instantaneous and specific agglutination of pneumococci in the circulation of infected rabbits. In all instances of both natural and passive immunity investigated, agglutination of the bacteria within the blood of the infected animal was followed by the rapid removal of the agglomerated cocci from the circulation and their destruction either by phagocytosis or in the capillary systems of the viscera. Unagglutinated pneumococci might remain in the blood stream and produce a progressive septicemia.

There could be found only one reference in the literature to the possible effect of an alien infection on agglutinins in the blood of

immunized animals. Reimann and Wu (1930)[1132] reported that experimental typhus fever in guinea pigs vaccinated with pneumococci had no influence on the pneumococcal agglutinins.

ADDITIONAL DATA

According to Goodner,[528] agglutinins are associated with the least soluble globulins—the euglobulin fraction—of antipneumococcic horse serum. No references could be found regarding the distribution of pneumococcal agglutinins in the protein portions of specific immune serum of other animals. In a study of the changes in bacterial volume as a result of specific agglutination, Jones and Little (1933)[681-2] stated that during specific agglutination globulin from immune serum is deposited on the surface of the organism. The increase in volume of pneumococci might be ascribed to the interaction of cellular carbohydrate and immune globulin—possibly agglutinin—which results in a swelling, or *Quellung* to use the Neufeld term, of the cocci.

In the mechanism of the type-specific agglutination of Pneumococcus, Francis[472] found that when the organisms were not present in sufficient numbers to absorb completely all the antibodies from immune serum, more antibody was bound by cellular carbohydrate than was required for the process of agglutination. The excess of antibody thus fixed could then unite with additional amounts of soluble specific substance when the polysaccharide was added in soluble form to the agglutinated material. When an excess of free SSS was added to an agglutinated mass of antibody and pneumococci, the organisms were redispersed, and in the suspended state were again specifically agglutinable. Francis concluded that the reactive substance of the pneumococcal cell in type-specific agglutination is the capsular polysaccharide. The observation agrees with the observation of Avery and Goebel (1933)[46] that Type I acetyl polysaccharide in purified form absorbs from Type I antipneumococcic serum all demonstrable type-specific agglutinins as well as precipitins and protective antibodies.

Precipitins

While investigating the massing of pneumococci in the presence of immune serum, Neufeld[974] dissolved the organisms in bile, added the clear solution to the specific serum which had agglutinated the culture, and noted that a particulate substance became microscopically visible within a quarter-hour. The aggregations grew and finally formed macroscopic masses of peculiar form and with a hyaline appearance. Neufeld thus showed the close relation that exists between specific agglutination and precipitation, and the experiments indicated that both phenomena were due to the same elements in the bacterial body and in the immune serum but, owing to the physical state of the antigenic substance, differed only in the manner of manifestation. In the next year (1903), Wadsworth,[1455] applying Neufeld's technique to saline suspensions of pneumococci and using normal rabbit bile for solution of the organisms, corroborated Neufeld's observations. In order to eliminate any action of the bile, Wadsworth shook the centrifuged cultures with strong salt solution, brought the suspensions to the isotonic point, and filtered them. The filtrate precipitated with immune rabbit serum as in the case of the bile solutions of the cocci. The experiment showed that the substance precipitable by immune serum was a constituent of the normal pneumococcal cell and that it could be extracted by suitable solvents. Panichi (1907),[1047] by using filtrates of broth cultures of pneumococci, obtained precipitation with serum from rabbits, sheep, and asses previously immunized with the same organism. The reaction in the different serums varied in degree. A marked reaction was characterized by an immediate opalescence; flakes soon separated, became larger, and settled to the bottom of the fluid in the form of a membrane which did not diffuse on shaking. In milder reactions the sediment was easily dispersed.

In 1917, Dochez and Avery[321] discovered that the urine of animals experimentally infected with Pneumococcus and also the urine and blood serum of individuals ill with lobar pneumonia

showed the presence of a specifically precipitable substance in almost every instance during some stage of the disease. Precipitinogen appeared as early as twelve hours after the initial chill and in some cases was demonstrable as late as five weeks following defervescence. The authors at the time presented data on the chemical nature of the soluble specific substance which showed that the substance which formed in the animal body during pneumococcal infection and passed through the kidneys into the urine was the complex carbohydrate which later was identified as the specific capsular polysaccharide.

A year later, Quigley,[1114] in following the precipitin reaction during the course of lobar pneumonia due to pneumococci of Types I, II, III, and Type (Group) IV, found that when the urine was tested at intervals of two to three days, the reaction gradually increased in intensity during a period of three or four days, persisted from two to nineteen days, and then gradually disappeared. There seemed to be no regularity as to the period in the disease when the precipitinogen appeared in the urine, nor as to the length of time it persisted. Furthermore, its presence seemed to be independent of crisis. In the same year Blake[125] reported that, in nineteen carefully studied cases, there was a definite relation between the excretion of soluble pneumococcal antigen in the urine and the development of antibodies in the blood in lobar pneumonia. Precipitins were found only in clinically mild cases with negative blood cultures and with no antigen in the urine; and these patients recovered. Blake stated that daily estimation of the concentration of soluble antigen excreted in the urine, taken with the number of cocci per cubic centimeter of blood, had great prognostic value in the individual case of lobar pneumonia.

The type-specificity of the precipitin reaction was verified by Blake (1917),[123] who employed as precipitinogen the peritoneal exudate from mice infected intraperitoneally with pneumococci of Types I, II, and III, and Group IV. The method applied to a large number of strains yielded consistently positive and specific re-

sults, the precipitate forming immediately without incubation when exudate and serum were of corresponding immunological type.

There exists more than presumptive evidence that antipneumococcal precipitin and agglutinin are the same substance. In a quantitative study of the precipitation and agglutination reactions, Heidelberger and Kabat[614] obtained results which seem to prove the truth of the assumption. After removing from a combined Type I and II antipneumococcic serum the species-specific antibodies by absorption with somatic protein and the C Fraction, it developed that the application of the quantitative agglutination method of Heidelberger and Kabat and the quantitative precipitation method devised by Heidelberger, Sia, and Kendall[630] and by Heidelberger, Kendall, and Soo Hoo[628] yielded figures, within the limits of the accuracy of the methods, practically identical for anticarbohydrate precipitin and agglutinin. Heidelberger and Kabat stated that while this relation held for unconcentrated serum, in purified antibody solutions somewhat more agglutinin than precipitin was found, which they considered might be due to alteration of a portion of the antibody in the process of purification. The quantitative correspondence of type-specific anticarbohydrate agglutinin and precipitin argues for the immunological and chemical identity of the two immune substances and supports the unitarian theory of antibodies originally formulated by Zinsser.[1578]

ANTIPROTEIN PRECIPITINS

The somatic protein of Pneumococcus possesses the property of stimulating the production of precipitin and of reacting with the antibodies so formed. Since the protein is common to pneumococci of all types and to degraded variants as well as to smooth, virulent forms of the organism, its precipitinogenic activity, as might be expected, is specific only for the species and not for type. These facts were reported by Avery and Morgan (1925),[54] who

found further that antiprotein serum failed to agglutinate type-specific strains of Pneumococcus or to react with the carbohydrate derived from them. A somewhat analogous reaction was described in the same year by Jungeblut.[698] When alcoholic extracts of washed, sedimented pneumococci of Types I, II, and III were mixed with tincture of benzoin, added to specific immune serum, and incubated at 40°, flocculation took place. Antipneumococcic serums of the three types with homologous antigens gave flocculation of varying intensity. The reaction was strictly specific for the bacterial species and at the same time might be highly type-specific.

SOMATIC CARBOHYDRATE (C FRACTION)

A phenomenon first discovered by Tillett and Francis[1409] and later investigated by Ash,[25] by Francis and Abernethy (1934),[475] by Abernethy and Francis,[2] and by Abernethy[1] still awaits an explanation.

It was found that the serum of individuals acutely ill with lobar pneumonia possesses the capacity of precipitating the somatic or C carbohydrate derived from pneumococci. It was further demonstrated that the precipitating action of patients' serum with the C Fraction is demonstrable in the early stages of pneumonia, sometimes within twenty-four hours of the onset, persists throughout the course of the active disease, or persists or recurs along with the development of complications, and disappears during convalescence.

In the report of the most recent experimental study available, Abernethy[1] stated that the serum of rabbits infected intradermally by the Goodner technique fails to precipitate the C carbohydrate, whereas the serum of monkeys (*Macacus cynomolgos*) given an intrabronchial infection with Type III Pneumococcus is capable of precipitating the somatic polysaccharide. The property is demonstrable within the first twenty-four hours following the experimental inoculation and persists for two or three days

during the period of active infection. With the recovery of the animal the reactivity of the serum disappears almost as abruptly as it appears with the onset of the disease. Abernethy reports that the study demonstrates:

. . . the variable response of two different hosts to the same bacterial agent. In one species, the monkey, pneumococcus infection was accompanied by the demonstration of certain changes in the serum during the acute period of illness. In the other, the rabbit, these changes were not observed. These observations suggest that during infection in the monkey either some newly formed substance or some alteration occurs in the serum which renders it inactive in precipitation tests with this particular polysaccharide derived from Pneumococcus. Assuming this to be the case, then the failure to demonstrate the phenomenon in the rabbit might be explained either by the absence of or qualitative differences in the serum during infection.

The phenomenon of precipitation of the somatic or C polysaccharide of Pneumococcus cannot be explained on the basis of any of our present orthodox conceptions of immunity. The property is not limited to the serum of individuals ill with pneumococcal infection, since it is also possessed by the serum of patients suffering from rheumatic fever, bacterial endocarditis, and lung abscess, but not from other febrile diseases.

Furthermore, unlike other specific antibodies, the substance capable of precipitating the C Fraction appears in the serum of the pneumonia patient within the first twenty-four hours of the disease, and—again unlike any antibodies that we know—disappears from the serum with the beginning of recovery. Because of their importance in the body's defense against pneumococcal and other infections, the discovery of the chemical identity of the precipitating substance in the serum of pneumonia patients and of the chemical or physical processes involved in the particulation of the somatic polysaccharide is awaited with keen interest.

Jungeblut believed that lipoid substances extracted from Pneumococcus were responsible for the reaction but, as the author pointed out, the method of preparing the alcohol-soluble antigen

did not preclude the possibility that certain impurities, protein or carbohydrate in character, might have been carried over into the extract. The lack of sharpness in type-specific action would denote the participation of cellular protein in the reaction.

A precipitin phenomenon described by Boor and Miller[138] in 1931 is yet to be explained. Nucleoprotein and non-protein fractions prepared from strains of Gonococcus gave precipitates when added to antipneumococcic serum of Types I, II, and III. The antigens were active in high dilutions, and displayed even greater precipitating power in the presence of antimeningococcic serum.

THE MECHANISM OF SPECIFIC PRECIPITATION

Gay and Chickering,[508-9] in 1914, found that the addition of a water-clear extract of pneumococci to homologous antiserum produces a voluminous precipitate which carries down with it agglutinins and the greater portion of protective antibodies. Subsequently, Chickering[223] found the reaction to be type-specific. In the reaction, therefore, it was evident that the precipitinogen consisted of some soluble component of the pneumococcal cell while the specific precipitin, as later shown by Goodner,[528] was a substance associated with the euglobulin of the horse serum employed.

Soluble specific substance. Heidelberger and Avery,[606] in 1923, isolated the soluble specific substance from Type II pneumococci and announced that it consisted mainly of a carbohydrate and that in as high a dilution as 1 to 3,000,000 gave positive precipitation with homologous antiserum. According to the authors, the precipitinogen was apparently a polysaccharide constituting the capsular substance of Pneumococcus. This important conclusion was the first evidence of a possible connection between the capsular material and the specific relationships of pneumococci, and accounted for the type-specificity of the precipitin reaction.

Felton and Bailey[420] fully substantiated the work of Gay and Chickering on the nature of the precipitate, and also the observation of Heidelberger and Avery[607] that a single component of

Pneumococcus—the soluble specific substance—was concerned in the particulation of type-specific antibody in immune serum.

Heidelberger, Goebel, and Avery (1925)[613] confirmed the participation of the soluble specific substance in immunological reactions and refined the methods for the isolation of the polysaccharide. The purified substance from Type I Pneumococcus gave a specific precipitin reaction with homologous Type I antipneumococcic serum and could be detected in a dilution as great as 1 to 6,000,000. Similar refined preparations from Type II and Type III pneumococci showed antigenic reactions of approximately the same magnitude, and in the precipitin reaction exhibited strict type-selectivity for the precipitins in homologous serum. The observations substantiated the view that the polysaccharides were the actual specific antigenic substances of Pneumococcus.

That the specific precipitinogen in Pneumococcus is evidently a soluble carbohydrate was shown in a different manner by Schiemann and Casper (1927),[1228] who dissolved pneumococci in sodium taurocholate, and found that the solutions gave type-specific precipitates with homologous immune serum. The authors rightly believed that the soluble carbohydrate obtained by lysis of the cocci with the bile salt was the same as the type-specific polysaccharide isolated and tested by Avery and Heidelberger.

Later (1931), Avery and Goebel[45] proved that the type-specificity of the interaction of Pneumococcus and homologous immune serum was due to the capsular polysaccharide and not to the protein of the cell. The serum of rabbits immunized with an artificial antigen, prepared by combining a specific derivative of the capsular polysaccharide of Type III Pneumococcus with globulin from horse serum, was found to contain specific precipitins for the Type III polysaccharide and, in addition, precipitins for horse globulin. It was, however, the capsular polysaccharide that was the determining factor in the specificity of the antigen as a whole. Subsequently, Avery and Goebel[46] proved that the acetyl polysaccharide, besides being antigenic in the sense of being capable of

stimulating the production of specific antibodies, was also anti-
genic in that, in a highly purified form, it reacted specifically with
homologous precipitin and completely removed precipitin from
immune serum.

Balance between precipitinogen and precipitin. Morgan (1923)[912]
demonstrated that a proper balance between antigen and anti-
body was required to bring about the phenomenon of precipita-
tion. In order to obtain the maximal precipitate from any given
quantity of antipneumococcic serum, a definite or optimal amount
of precipitinogen or soluble specific substance was necessary. The
ratio between the quantity of immune serum and reacting sub-
stance was found to be practically constant. Therefore, when an
excess of precipitinogen was present, the resulting precipitate
was not only decreased in amount, as determined by inspection and
weight, but its appearance was altered. The importance of the
relative proportions of antigen and antibody was also demon-
strated by Dean and Webb (1926)[311] who, employing normal
horse serum and its homologous antiserum, devised a method by
which it was possible to determine quantitatively the amount of
antigen and antibody and therefore the optimal ratio between the
two components necessary for complete precipitation.

Precipitin index. Sobotka and Friedländer (1928)[1299] deter-
mined that the sensitivity of the precipitin reaction with anti-
pneumococcic serum could be expressed by the product of the con-
centrations of the two reacting substances, antigen and antibody,
and defined the precipitin index (P.I.) as one-millionth of the re-
ciprocal value of the product. In investigating the zonal phenome-
non and its bearing on the absolute concentration and equivalent
weight of antibody, the authors found that the greater tendency
toward the exhibition of a post-zone in Type III precipitation
was connected with the lower acid equivalent .of the homologous
capsular polysaccharide. The addition of normal serum as well as
an increase in pH promoted the post-zonal effect. By determining
the precipitin index, it was possible to recognize and to eliminate

zonal irregularities. Smith[1298] later (1932) applied the principle of optimal proportions to precipitin tests with Type I soluble specific substance and homologous antibody and reported that the method gave a true index of the protective power of the serum. More recently Felton devised a test for the combining power of antipneumococcic serum with specific polysaccharide.

In the description by Francis,[472] already cited in the discussion of agglutinins, it appeared that differences in the ratio of antigen and antibody which affect the agglutination reaction apply also to the precipitation reaction. When a solution of soluble specific substance was added in excess to homologous immune serum, a pro-zone was created in which precipitation was inhibited. As in the agglutination reaction, Francis showed that it was the capsular polysaccharide which constituted the precipitinogen.

Quantitative relations in precipitation. In 1929, Heidelberger and Kendall[616] published the first of a series of communications on a quantitative study of the precipitin reaction as related to soluble specific substance and homologous immune serum. After precipitating Type III antipneumococcic serum with Type III polysaccharide, the authors measured the amount of nitrogen in the supernatant fluid of the precipitated mixture, and then from the nitrogen content calculated the amount of protein remaining in solution. The authors then tested the experimental data by the law of mass action to determine whether the reaction showed analogies to the behavior of simpler ionic reactions. Depending on the relative amounts of the reactants, the specific precipitate appeared to be a mixture of varying proportions of two compounds, or a whole series of compounds, containing hapten and antibody in varying proportions, whose limits could be expressed by the following equilibriums, A and S being respectively equivalent amounts of antibody and Type II soluble specific substance:

$$(1) \quad A + S \rightleftharpoons AS \ (120:1 \ ratio)$$
$$(2) \ AS + S \rightleftharpoons AS_2 \ (60:1 \ ratio)$$

According to Heidelberger and Kendall, an inhibition-zone effect appears to be a chemical equilibrium which might be expressed by

$$(3)\ AS_2 + S \rightleftharpoons AS_3$$
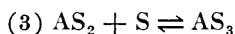
In equilibrium (1) the reaction tends to proceed strongly to the right since AS is only slightly soluble. Reaction (2) will go farther to completion and more AS_2 will be precipitated (since AS_2 has an appreciable solubility and dissociation tendency) when a little S is added after equilibrium (2) has been reached. When much S is added, equilibrium (3) comes into play and the precipitate dissolves. The authors suggested as an analogy the reaction between silver and the cyanide ion, where a small amount of CN^- causes a precipitate of AgCN, and an excess of CN^- causes the formation of the silver cyanide complex which is soluble.

Heidelberger, Sia, and Kendall (1930)[630] described a rapid and simple method for the approximate determination of the specifically precipitable protein in Type I antipneumococcic serum. Inasmuch as a close parallel was found to exist between the amount of specifically precipitable protein and the number of mouse protection units, and because of the rapidity, simplicity, and economy of the method, the authors proposed its use instead of the mouse protection test as a basis for the titration of standard serum and for the comparison of other serums with the standard.

Heidelberger and Kendall (1932)[619] devised a method, based on the precipitin reaction, for the microdetermination of the specific polysaccharide of Type III Pneumococcus. By the method, as little as 0.01 milligram of Type III polysaccharide could be measured, while the procedure appeared to be applicable to any specific polysaccharide upon standardization of an homologous antiserum or antibody solution in the region of excess antibody. In another paper,[620] the authors described precipitin reactions performed with partial hydrolytic products of the specific capsular polysaccharide of Type III Pneumococcus freed quantitatively from unhydrolyzed specific polysaccharide. The fractions

yielded specific precipitates, in dilutions of 1 to 1,000,000, with Type III antipneumococcic horse serum, but failed to precipitate homologous rabbit antiserum, giving rise to specific inhibition. The authors found further that aldobionic acid, the structural unit of Type III polysaccharide, did not precipitate homologous antiserum.

With Soo Hoo, Heidelberger and Kendall (1933)[628] described a method, developed from the previous work of the two last-named authors, for the microestimation of precipitin in antiserum. The technique involved the use of a deep-red protein dye, the R salt of azo-benzidene-azo-crystalline egg albumen. The procedure gave the actual weight of precipitin and could be applied to the maximal amount of precipitable antibody in any antiserum. The observation that four samples of immune rabbit serum produced in response to injection of the dye contained over one hundred times as much precipitin as the antigen injected, appeared to the authors as supplementing the growing mass of evidence against the theory that specific antigen fragments are actually incorporated into the antibody molecule.

In 1935, Heidelberger and Kendall[622] pointed out that the usual immunological technique, namely, incubation of precipitin reactions at 37° for two hours and allowing the tubes to stand in the ice-box over night, while resulting in the maximal precipitation of antibody from immune rabbit serum, in the case of antipneumococcic horse serum or purified antibody, does not permit the establishment of a true equilibrium or the precipitation of the maximal amount of antibody nitrogen. The authors recommended that analyses of antipneumococcic horse serum should therefore be carried out at 0° and the tubes containing the antigen and immune serum should be allowed to stand in the cold for at least twenty-four hours in order to ensure the completion of the reaction.

In an accompanying paper, Heidelberger and Kendall[623] reported the results of additional study of the quantitative relations in the precipitin reaction between Type III polysaccharide and

homologous immune horse serum, which led the authors to the con-
clusion that the reaction could be accounted for quantitatively by
assuming the chemical combination of the components in a bimo-
lecular reaction, followed by a series of competing bimolecular
reactions which depend on the relative proportions of the com-
ponents. From the data, Heidelberger and Kendall then developed
additional mathematical formulas representing the developments
in the mechanism of the precipitin reaction. As the authors re-
marked in their 1929 communication, "Of all the reactions of im-
munity the precipitin test is perhaps the most dramatic and strik-
ing; while other immune reactions are more delicate, the precipitin
test is among the most specific and least subject to errors and
technical difficulties"; and "The isolation of bacterial polysac-
charides which precipitate antisera specifically and possess powers
of haptens has not only afforded one of the components of a pre-
cipitin reaction in a state of comparative purity, but has greatly
simplified the analytical problem." By these exact quantitative
methods, the precipitin reaction has conferred inestimable benefits
on the immunologist in understanding the nature of immunological
phenomena, such as the relations between antigen and antibody,
in judging the purity of antigen, and in estimating the antibody
content of antipneumococcic serum.

The chemical study of capsular polysaccharides and pneumo-
coccal antibody is furnishing clues to the process which operate in
the union between type-specific antigen and homologous antibody.
By simple chemical experiments, Chow and Goebel[226] showed that
the immunological activity of pneumococcal antibody protein is
to a great extent dependent upon the presence of free amino
groups in the protein molecule. With the work of Landsteiner and
van der Scheer[782-3] in mind and reasoning from the results of the
study on synthetic antigens made by Avery and Goebel (1929),[44]
Chow and Goebel assumed that in the case of the type-specific anti-
body of Pneumococcus the spatial arrangements of the polar

groups in the immune protein may determine its specific capacity to react with the polysaccharide of the homologous type.

In the carbohydrate of Type I Pneumococcus the authors believed, on the basis of the experimental evidence, that the carboxyl groups of the polysaccharide are the dominant groups which interact to form the immune precipitate. The questions whether the carboxyl groups of the polysaccharide actually combine with the amino groups of the antibody protein and whether the formation of an insoluble precipitate involves further chemical change in the protein molecule, such as specific denaturation, cannot be answered at present. However, Chow and Goebel concluded:

The specificity of this reaction is determined by the stereochemical relationship of the dominant polar groups in the reacting molecules, whether they be antigen or antibody. If the spatial pattern of the polar groups of both antigen and antibody is of exactly the correct order, then union occurs. If, however, this relationship is disturbed by artificial means, as has been experimentally demonstrated by covering the dominant polar group of either polysaccharide or antibody with a chemical radical, the pattern is destroyed and union between them is either greatly modified or fails to take place. When the original constitution of the reacting substances is restored, however, serological specificity is regained.

CORRELATION OF PRECIPITINS WITH OTHER ANTIBODIES

Many observations have been made on the quantitative relationships existing between precipitins and other specific antibodies in antipneumococcic serum. The discovery that the capsular polysaccharide functions as the precipitinogen in the reaction, and the development of methods for evaluating the strength of antigen and antibody, have made possible more exact determination of the amount of precipitin in a given serum in relation to the content of other immune substances. Friedlander, Sobotka, and Banzhaf (1928)[493] estimated, under known conditions, the precipitin indices of a number of monovalent and polyvalent antipneumococcic

serums and found the indices to vary as did the number of pro-
tective units. The ratio of the precipitin index to protective units
in monovalent serums was between 2.8 and 4.8 for Type I and
about ten times greater for Type III. Lower values prevailed in
polyvalent antipneumococcic horse serum and in mixtures of
heterologous monovalent serums. A relative increase in precipitin
activity was found in the refined and concentrated serums tested.

The experiments of Avery and Goebel (1931)[45] with the arti-
ficially conjugated Type III capsular polysaccharide-horse serum
globulin would seem to argue for the unity of type-specific pre-
cipitins, agglutinins, and protective antibodies.

In a systematic study of the quantitative relations existing be-
tween the various specific antibodies in Type I and II antipneu-
mococcic horse serum, Felton (1931)[407] determined for Type I
Pneumococcus the correlation coefficient between protective and
precipitin titer as 0.93; between protection and agglutination the
figure was 0.80; between protection and neutralization it was
0.88; and between protection and the amount of protein precipi-
tated with specific carbohydrate it was 0.91. From this degree of
correlation it appeared to Felton that, at least for freshly drawn
horse serum, the precipitin test could be used to estimate the prob-
able therapeutic value of antipneumococcic serum.

The qualification made by Felton in respect to the freshness of
the serum sample as influencing precipitation and protective titer
was confirmed by Valentine, McGuire, Whitney, and Falk (1931)[1443]
in experiments on the effect of ageing on antibodies in dried anti-
pneumococcic serum. At room temperature, agglutinin and pre-
cipitin titer decreased to a greater degree than did the number of
mouse-protective units, although the deterioration of the two first-
named antibodies failed to take place at ice-box temperatures.

In a more recent publication (1936), Barnes, Clarke, and
Wight[82] compared the unit value of serums obtained by mouse
protection with a) the water test; b) agglutination test; c) the
authors' routine precipitation test; d) the optimal proportions

precipitation test; e) Felton's test for combining equivalents; and f) a modified precipitation test devised by the authors. The following table taken from page 131 of the authors' communication shows the correlations found between precipitation, combining equivalents, agglutination, and mouse protection.

Method of titration	Serums tested with control		Incubation		End-point = highest dilution showing	Antigen	Correlation with mouse protection
	P 11	P 403	Hours at 40°	Ice-box			
Agglutination	32	18	2	Over-night	Trace of aggluti-nation	Live broth culture	0.713 ±0.047
Optimal propor-tions precipita-tion	0	50	*	*	*	SSS	0.909 ±0.016
Routine precipita-tion	50	0	2	Over-night	Definite floccu-lation	SSS 1:10,000 dilution	0.866 ±0.024
Modified routine precipitation ...	50	0	2	Over-night	Definite floccu-lation	SSS 1:10,000 dilution	0.930 ±0.012
Combining equiva-lent	50	0	*	*	No un-com-bined SSS	SSS 1:10,000 dilution	0.925 ±0.014
Water test	0	50	0	Over-night	*	*	0.652 ±0.055

* Not applicable.

By closely spacing the serum dilutions in the modified test, Barnes, Clarke, and Wight succeeded in obtaining precipitin titers which agreed more closely with estimations of the mouse-protective value than with any of the other methods tested. It would seem, therefore, that the precipitin content, as determined by this method, is in close agreement with the amount of protective antibody in specific antipneumococcic serum.

Complement-Fixing Antibodies

The reaction of complement fixation is so far inferior in delicacy to that of agglutination, precipitation, or mouse protection for the serological diagnosis of pneumococcal types that the subject can be dismissed with brief mention. Hanes (1914),[588] using the method, found that strains of *Pneumococcus mucosus* were more closely related to Pneumococcus than to Streptococcus. There was a certain amount of cross-fixation between immune serum of Types I and II, and Type III antigens, whereas antistreptococcic serums deviated complement only in the presence of antigens made from homologous strains. Christensen (1922)[228-9] employed the method of complement fixation to check type-determination of pneumococci of the first four types made by the agglutination reaction but, because the first-named method was more complicated and required a longer time for its execution, Christensen did not recommend its use in place of agglutination. In the studies made by Avery and Heidelberger (1925)[49] on the antibodies demonstrable in the serum of rabbits immunized by injection of intact pneumococci and of their carbohydrate and protein derivatives, the method of complement fixation yielded results agreeing with those obtained by specific precipitation. Bull and McKee (1929)[180] applied the method to test the production of antibodies in rabbits immunized with heat-killed broth cultures of pneumococci. Serum from animals immunized with a Type II strain fixed complement in the presence of antigens of Types I, II, and III, but the titer was always higher with the homologous antigen. The same observation held true for Type III antiserum. From rabbits injected with a Type (Group) IV organism, the serum was potent when the homologous organism was used as antigen and only slightly weaker in the presence of Type I antigen. Although the serums were low in agglutinin titer, their action was type-specific.

The mechanism of the complement-fixation reaction with the components of Pneumococcus, and the disparity between the abil-

ity of specific antipneumococcic serums produced respectively in
the rabbit and horse to bind complement in the presence of homolo-
gous antigen, have been studied by Goodner and Horsfall.[538] With-
out retailing the experimental details, it may suffice to abstract
the discussion and summary. The experiments reported supported
the view that the failure to obtain complement fixation with com-
binations of pneumococcal capsular polysaccharide and specific
immune horse serum is not due to some heterologous inhibitor in
immune horse serum but is to be referred rather to some property
of the horse antibody itself or some property of the immune ag-
gregate resulting from the union of this antibody and the poly-
saccharide. This property is lacking in the specific antibody in
immune rabbit serum which, in the presence of homologous poly-
saccharide, is capable under proper conditions of binding com-
plement. Furthermore, the results support the view that the fixa-
tion of complement is a phenomenon of selective absorption. That
one type of aggregate absorbs complement while another fails to
do so is curious, but far from unique. Goodner and Horsfall found
a close parallelism in the fact that horse antibody-polysaccharide
aggregates absorb cephalin, while aggregates containing rabbit
antibody selectively absorb lecithin.

The essential role of serum lipids in the demonstration of the
phenomena of specific precipitation and agglutination has been
described by Horsfall and Goodner.[657] The removal of lecithin
from antipneumococcic horse serum and, to a lesser degree, the ex-
traction of cephalin from antipneumococcic rabbit serum cause a
loss of the visible phenomena of agglutination and precipitation.
It was found that initial activity of type-specific antibody can be
restored to extracted immune horse serum by the addition of leci-
thin, and to extracted rabbit serum by the addition of cephalin. It
is therefore probable that the content of these two phospho-lipids*
in the serum of the two animal species may, in part, account for

* In a recent brief communication, Horsfall, Goodner, and MacLeod[659] de-
scribe the antibody in pneumococcal immune horse serum as a lecithoprotein
and that in immune rabbit serum as a cephaloprotein.

the differences in the immunological behavior of immune horse and rabbit serum.

Inasmuch as complement fixation does not occur in the absence of particulation and since particulation is a secondary phenomenon in the reaction, the authors regard complement fixation as a tertiary manifestation.

The evidence presented in the communications cited and in other reports is sufficient to demonstrate that the method of complement fixation offers no advantages over other serological methods for the identification of pneumococci or of types within the species.

Bactericidins

With the advancement of bacteriological knowledge, the pneumococcidal action of both normal and immune serum has been found to be a more complicated process than it was earlier conceived to be. Instead of being solely a function of possible bactericidins in serum, the destructive effect of blood involves the action not only of serum but of leucocytes and of fixed tissue cells. Since the pneumococcidal power of blood depends on the associated participation of tropins or opsonins and phagocytes, the special features of their combined action will be described later in the present chapter, while the nature of other inherent defenses of the animal body will be discussed in a subsequent chapter.

Antihemotoxin

Experiments on the antigenic action of pneumococcal hemotoxin reported in 1914 by Cole[252] indicated that the serum of rabbits and sheep immunized with hemolytic extracts of Pneumococcus had acquired increased power to inhibit the lytic effect of the extract on erythrocytes. In studies on the oxidation and reduction of immunological substances, Neill[952] and his colleagues[958-9] compared the immunological response to the injection into rabbits of reduced and oxidized pneumococcal extracts, and succeeded in producing a neutralizing antibody by immunizing the animal with

the hemolytically inactive hemotoxin present in oxidized solutions as well as with the active hemotoxin. The antibody appeared to be a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci, since it was without effect on the hemotoxins of tetanus and Welch bacilli. Neill with Fleming and Gaspari determined that for the production of antihemotoxin in the rabbit or horse it was essential that the hemotoxic antigen be used in an unheated condition.

A similar antihemotoxic serum was developed by Cotoni and Chambrin (1928)[282] in rabbits, sheep, and horses after immunizing the animals with living cultures, extracts of living and dried pneumococci, as well as with dried organisms killed by alcohol and ether. The antihemotoxin was stable after heating for one-half hour and, as Neill[950] found, was devoid of type-specificity but possessed species-specificity, because when tested against the hemolysins of streptococci, tetanus bacilli, septic vibrios, and *Bacillus perfringens*, the serum displayed no neutralizing action. Doubt was cast on the species-specificity of antihemotoxins by Todd (1934),[1412] who apparently was able to neutralize, to a limited extent, the hemolysins of Type II and Type III pneumococci with antistreptolytic serum. The degree of neutralization was not necessarily correlated with the antistreptolytic titer, and the different hemolysins could be distinguished by quantitative serological methods. The partial antigenic overlapping of hemolysins could only be demonstrated by the use of hyperimmune serum.

Antitoxin

Boehncke and Mouriz-Riesgo (1915),[134] notwithstanding the fact that they were never able to obtain any very active pneumococcal toxin, believed that the curative action of some of the immune serum prepared by them was due to its antitoxic qualities. Olson (1926)[1029-30] claimed that it was possible to immunize mice by serial injections of pneumococcal toxin prepared by allowing sodium ricinoleate to act on pneumococci, but the author pre-

sented no serological evidence of the presence of antitoxin in the serum of the immune animals. In addition, serum from normal horses, sheep, rabbits, and chickens, as well as antipneumococcic serum or pneumococcic antibody solution, was found to possess little power to prevent the lung changes or cutaneous reactions evoked in mice by the toxin.

Clowes, Jamieson, and Olson (1926),[244] by injecting rabbits, sheep, and horses with progressively increasing doses of sterile toxic extracts prepared by the sodium ricinoleate method of Larson, obtained a serum that neutralized the skin-reacting substance contained in the extracts, and suggested as a provisional unit of antitoxin the amount of serum required to neutralize one million skin-test doses of toxin. Concentration of the immune serum effected the removal of 99.9 per cent of the total serum-protein without appreciable loss of antitoxin. Larson,[788] in the same year, administered an immune serum prepared and tested in the same manner to patients ill with lobar pneumonia and, because the patients showed a rapid drop in temperature and experienced relief of subjective symptoms following the administration of the serum, he believed that the serum actually contained antitoxin.

The work of Parker (1929),[1061] referred to earlier in the text, has a definite bearing on the question of the possible existence of pneumococcal antitoxin. Serum prepared by the author in rabbits and horses by using sterile filtrates of pneumotoxin as antigens, under certain conditions, protected guinea pigs against the pneumonia caused by the intratracheal injection of living pneumococci and toxic pneumococcal autolysates. The protection thus conferred was heterologous for type and appeared to be due to some immune substance other than protective antibodies, since the latter could not be demonstrated in the active serum of rabbits and horses immunized with sterile filtrates of toxic autolysates of Pneumococcus. In a second publication, Parker and McCoy (1929)[1062] described a method for standardizing the potency of antitoxic horse serum and set as the unit of toxin the amount of

filtered autolysate which, when injected intratracheally, would kill a guinea pig weighing 200 to 210 grams in from four to twenty-four hours with typical symptoms and necropsy findings; while one unit of antitoxin represented the smallest amount of serum necessary to protect a guinea pig of the same weight against one unit of toxin when the test toxin-serum mixture was injected intratracheally. Neither normal horse serum nor antipneumococcic horse serum containing 500 protective units per cubic centimeter when used in a 1 to 10 dilution exerted a detoxifying action on the toxin. A sample of antipneumococcic serum concentrated by the Felton method, in a 1 to 20 dilution, neutralized the toxin but failed to do so when added in a 1 to 50 dilution.

The results obtained by Jamieson and Powell (1931)[676] were analogous to those reported by Parker and McCoy. The filtrates of young broth cultures of pneumococci of Types I, II, III, and IV, which had been found to elicit a positive reaction in the skin of rabbits and of human beings, constituted the antigen employed by subcutaneous injection for immunizing horses. The serums developed by the procedure appeared to possess neutralizing substances for the toxin, and these substances could be concentrated to a moderate degree in the refining of globulins by the usual salting-out methods. The concentrated serum contained only a small amount of protective antibody, and in neutralizing action compared favorably with the action of specific antitoxin on scarlet fever streptococcal toxin.

Employing autolysate prepared by the method of Parker, Blackman[122] produced immune serum in rabbits and horses that would protect normal rabbits not only against the toxic action of autolysate but against pneumococcal infection.

Sabin (1931)[1203] sought to determine whether antipneumotoxin influences the course of pneumococcal infection in mice. Mice were injected with large doses of Type I and II pneumococci. One series of animals was then treated with therapeutic antibacterial serum, another with antipneumotoxic serum, and a third series re-

ceived both antibacterial and antitoxic serums. The results showed that the antitoxin was without effect in staying the infection. Sabin then tested the action of antipneumotoxin in rabbits infected intradermally by the method of Goodner. Rabbits were given an intradermal injection of 0.1 cubic centimeter of a 1 to 100 dilution of an eighteen-hour broth culture of virulent Type I Pneumococcus. Three groups of rabbits so injected were treated with the same serums as in the mouse experiments, some animals receiving the serum injection six or seven hours after inoculation and some twenty-four hours after inoculation. Of the untreated controls and those animals receiving antitoxin only, all died within the same time, and no beneficial effect was observed in the rabbits treated with antitoxic serum added to antibacterial serum.

The preliminary statement of Coca (1932)[245] regarding the antigenic action of a pyrogenic and skin-reacting substance in culture filtrates of Pneumococcus in raising the resistance of children to the action of the antigen has already been mentioned. Not only did the young subjects become immune to the pyrogenic substance and fail to react when the filtrate was injected into the skin, but the serum of the children so treated neutralized the action of the filtrate. Convalescent serum from patients recovering from infection with Type I and II Pneumococcus also inhibited the effect of the poisonous substance derived from pneumococci. Therapeutic antipneumococcic serum, on the contrary, in the amounts used, failed to neutralize minute amounts of the filtrate.

In a more recent report (1936), Coca[246] described further experiments with filtrates of pneumococcal cultures. The toxic principle was neutralized by the serum of young human subjects previously injected with the filtrates and by the serum of patients convalescing from lobar pneumonia. According to Coca, the neutralizing action of the human immune serum was type-specific and antitoxic in nature and not related to the type-specific polysaccharide antibody.

Heterophile Antibodies

During recent years there has developed considerable interest
in the fact that the injection of pneumococci into suitable animals
results in the production of heterophile antibodies. It is not neces-
sary in this discussion to consider all the various sources and char-
acters of heterogenetic antigens which may be used to stimulate
the production of heterophile antibodies. Readers interested in the
subject are referred to reviews of the subject by Davidsohn
(1927),[294-7] Bull (1928),[175] and more recently and in special ref-
erence to pneumonia, by Plummer (1936).[1099] That heterogenetic
antigens and heterophile antibodies may be of biological signifi-
cance in pneumococcal infections has been suggested in publica-
tions by Bailey and Shorb (1931, 1933),[64-5] and by Powell, Jamie-
son, Bailey, and Hyde (1933).[1105] Opposed to these views are the
observations of Finland, Ruegsegger, and Felton (1935).[444] It is
not proposed to relate technical details of the various experiments
dealing with this subject. The Council on Pharmacy and Chemis-
try of the American Medical Association requested Plummer to
make a report on the use of heterophile antibodies and, since his
conclusions appear sound, they are reproduced verbatim.

The presence of heterophile bodies in animal tissues, animal serums
and bacteria is strongly suggestive that these bodies play a part in cer-
tain immune reactions. The animal experiments carried out by Bailey,
Shorb, Powell, Jamieson, and Hyde do not prove that the heterophile
bodies play a role in the pneumococcus immunity of the human being.
Some of the results reported are open to question. The work should be
repeated by an independent group of investigators before it is accepted.
The study by Finland, Ruegsegger and Felton on the heterophile anti-
bodies in the serum of patients convalescing from pneumonia and in
controls leads one to believe that the heterophile bodies do not have any
particular bearing on the course of pneumococcic infections in man.
The clinical and statistical evidence cited by the manufacturers of the
combined heterophile antibody serum is too limited and is strongly mis-
leading. The presence of both rabbit and horse proteins in the serum
will increase the incidence and the dangers of allergic reactions. This is

a definite disadvantage, because with newer methods of refining serum the allergic reaction is the principal source of danger in any type of serotherapy. There is only slight experimental evidence and no clinical evidence that the combined heterophile serum gives any immunity against Type III and Group IV pneumococcic infections. There is the same lack of evidence that the combined serum produces a greater immunity for Type I and II infections than the ordinary antipneumococcus horse serum. The principal theoretical advantage of the combined heterophile serum is that it could be used for all pneumococcic pneumonias, regardless of types, and the corollary of this that pneumococcus typing would not be necessary. Serum having this advantage would be highly desirable, but because its superiority is unproved and because of the probable increase in allergic reactions it is unfair and unwise to recommend the combined heterophile serum for general distribution.

The Council on Pharmacy and Chemistry approved and adopted Plummer's report and emphasized the conclusion that, in the light of present knowledge, recommendation of the combined heterophile serum for general distribution is unwise and unwarranted.

Phagocytosis

The part played by leucocytes in checking the invasion of pneumococci and in the destruction of cocci in the infected body was apparently first suggested by Gamaléia[498] in 1888. The French author, in a study of pneumonia patients, noted large phagocytes packed with cocci in various stages of degeneration. The author also reported an observation to the effect that sheep infected intratracheally with diplococci obtained from infected cadavers, on being sacrificed, showed many phagocytes containing the organisms. Gamaléia ventured the conjecture that pulmonary phagocytes were a factor in restraining the spread of pneumococci (*Streptococcus lanceolatus Pasteuri*) in the lung. Kruse and Pansini (1891)[763] also noted phagocytosis in animals experimentally infected with pneumococci, but held that the phenomenon was of secondary importance to the bactericidal action of the serum.

At the time, opinion was divided concerning the nature of the

forces which accounted for the resistance of normal and immunized animals to Pneumococcus. One school contended that immunity was due to bactericidins; the other maintained that the immunity was antitoxic in nature. The idea that mobile cellular elements— the leucocytes—might intervene in arresting pneumococcal infection came as an innovation and at first was not readily accepted. For example, Bonome[187] shared the conception that the blood of immunized animals acquired increased bactericidal power for pneumococci and although he observed both leucocytosis and phagocytosis, he did not perceive that the phenomena could be the basis of bactericidal action. It was Issaeff (1893)[673] who, as a result of his experiments, abandoned the idea that antitoxin was a factor, and emphasized the fact that phagocytosis played a most important part in acquired immunity to Pneumococcus. Mennes (1897)[893] was unable to demonstrate any effect of the white cells of normal blood on the development of pneumococcal infection. According to his views, the primary defensive element was the serum and not the leucocytes. The white cells of immune rabbits, however, exerted marked phagocytic action, and Mennes concluded that the immunity of the rabbit to pneumococcal invasion developed from a modification of the serum, and that the modification activated the phagocytic property of the leucocytes.

In 1904, Neufeld and Rimpau[997] definitely discarded bactericidins and bacteriolysins as forces operating in antipneumococcic immunity and reported that the addition of specific immune serum to normal rabbit leucocytes imparted to the cells vigorous phagocytic ability. The authors then extended the experiments to include the *in vivo* action of immune serum on virulent cultures injected into mice. When the culture alone was injected intraperitoneally, the organisms multiplied and only a few leucocytes were seen to contain cocci, but when both culture and serum were injected, a large number of pneumococci were engulfed by the white blood cells. By absorption experiments, Neufeld and Rimpau demonstrated that the serum acted on the cocci, whether living or killed,

and not on the leucocytes. Complement was not necessary to render the organisms phagocytable. In a second report, Neufeld and Rimpau (1905)[998] claimed that the theory that serum was a stimulant for the leucocytes was no longer tenable, but that serum, on the contrary, caused a peculiar transformation of the bacteria. Immune serums which possessed the property of rendering bacteria susceptible to phagocytosis Neufeld and Rimpau designated by the name "bacteriotropic" to distinguish them from bacteriolytic serums.

In America, at the time when the Wright opsonic technique was arousing such general interest, Rosenow[1160] tested the susceptibility of forty strains of pneumococci to phagocytosis. All but four strains at first resisted phagocytosis in pneumonic blood, but after cultivation on artificial media the organisms became susceptible to leucocytic ingestion. While heating the cocci had no effect on susceptibility, the opsonic property of the serum appeared to be diminished after a thirty-minute exposure to a temperature of 56°. Rosenow believed that white cells from the blood of pneumonia patients possessed greater phagocytic power than did normal cells, and ascribed the pneumococcidal effect of blood to the combined action of serum and leucocytes—phagocytosis and intraphagocytic digestion. Potter and Krumwiede (1907)[1103] tested the leucocytes of pneumonia patients for phagocytic properties. While granting the inaccuracy of the method employed, the authors, contrary to Rosenow, stated that leucocytes of patients during the height of pneumonic disease were probably less active in phagocytic power than were normal leucocytes.

The principle in leucocytes responsible for the lysis of pneumococci after being phagocyted was investigated by Schneider (1910),[1244] who believed that the hypothetical substance acted directly upon the injected pneumococci. Schneider also found that specific immune serum powerfully stimulated phagocytosis, especially *in vivo*, but ascribed the action of serum to its effect on the white cells. Schneider further claimed that a serum which was

lacking in this stimulating property was also lacking in precipitating, agglutinating, and complement-fixing action and displayed no protective power. Boehncke and Mouriz-Riesgo (1915),[134] who had previously corroborated the conclusion of Neufeld and Rimpau that the bacteriotropic action of antipneumococcic serum *in vitro* also participates in the action of the serum *in vivo*, were not convinced that tropins were the only factor in inducing phagocytosis. The authors had been able to rule out any participation of bactericidal and complement-fixing antibodies and, while they concluded that phagocytosis was the most important factor, the authors still inclined to the view that antitoxins might play an important part in resistance to pneumococcal infection. In 1919, Barber,[77] employing the single-cell technique, sought an explanation of the ready growth of pneumococci in immune serum. Failing to find any specific bactericidal substance in immune serum, Barber noticed that when specific serum was added to pneumococci previous to mixing with leucocytes active phagocytosis took place, and the same phenomenon occurred when the organisms were injected into the peritoneal cavity of mice passively immunized with immune serum. The author offered no explanation for the reaction.

SENSITIZATION OF PNEUMOCOCCI

Bürgers,[188] and Bürgers and Meisner (1911),[189] following the lead furnished by the work of Neufeld and Rimpau, found that while normal serum was inactive in rendering virulent pneumococci phagocytable, immune serum was highly active in this respect. Although the serum used in the experiments was evidently low in potency, the pneumococci when incubated with the serum were readily taken up by normal leucocytes. Bürgers and his colleague believed that fresh complement was necessary for phagocytosis, since they considered that the reaction was one depending upon antigen, amboceptor, and complement. Strouse (1911)[134b] also used the method of sensitizing pneumococci in studying the pres-

ence of immune opsonins in the serum of pneumonia patients. The addition of the serum accelerated the ingestion of pneumococci by the white cells.

In 1915, Friel[494] described the action of normal and immune serum on pneumococci. Cultures left in contact with normal serum for one and one-half to twenty-four hours and then washed in salt solution acquired no increased susceptibility to the action of phagocytes, but organisms similarly treated with immune serum were avidly ingested by the white cells. It will be recalled that the sensitizing effect of immune serum was called "piantication" by Friel. The term, meaning to prepare for slaughter, was in effect the same as Wright's word opsonization, meaning to prepare for the feast. In addition to the action of opsonins in rendering pneumococci susceptible to the destructive action of leucocytes, Goodner, Dubos, and Avery (1932)[536] demonstrated that organisms denuded of capsule by the action of specific bacterial enzymes became highly vulnerable to phagocytosis by tissue cells.

In an endeavor to clarify the contradictory conceptions of Neufeld and Rimpau[997] on the one hand and of Römer* on the other regarding the action of immune serum on bacterium or leucocyte, Preisz (1915),[1108] by employing normal and immune serum and leucocytes in both *in vitro* and *in vivo* experiments, concluded that the ability of immune serum to promote phagocytosis of pneumococci lay in its action on the cocci and not on the leucocytes.

A more recent report—that of Robertson and Sia (1927)[1147]— dealt with the action of the serum of both naturally resistant and susceptible animals on the phagocytability of Pneumococcus. When virulent cultures were sensitized by contact for one hour at 37° with the serum of animals resistant to Pneumococcus, the organisms were actively ingested not only by homologous leucocytes but also by the white blood corpuscles of other resistant and susceptible animals. On the contrary, when pneumococci were exposed to the action of the serum of animals normally susceptible to pneu-

* Quoted by Neufeld and Haendel.[992]

mococcal infection, the cocci were not taken up by the leucocytes of either resistant or susceptible animals. The serum of all the resistant species tested—dog, cat, sheep, pig, and horse—possessed marked opsonic properties not found in the serum of animals of susceptible species, such as the rabbit, guinea pig, and man. There appeared, however, to be no essential difference in the phagocytic activity of the leucocytes from the various animals. Heating the serum, according to Robertson and Sia, abolished the destructive power of serum-leucocyte mixtures for pneumococci. The validity of the authors' classification of resistant and susceptible animals is open to question. To group the horse with such highly resistant animals as the dog, cat, sheep, and pig, and to include species of such diverse susceptibility in the non-resistant class, may not be wholly justified. In another portion of the report, Robertson and Sia drew attention to other conditions which may influence the opsonizing property of serum on pneumococci subjected to the action of leucocytes. The strain and type of the culture employed, the age of the animals, and other factors discussed in the chapter on Pathogenicity must be taken into account in evaluating the results.

From the conclusions in an extensive report on an investigation of experimental pneumococcal septicemia and antipneumococcal immunity published by Wright in 1927,[1547] certain passages may be cited. Avirulent pneumococci of Type I inoculated intravenously into rabbits are rapidly removed and do not reappear in the blood; virulent strains of the same serological type similarly inoculated disappear for a short period and then subsequently increase in number. Previous active immunization enhances the capacity of the rabbit to remove virulent organisms and to prevent their reappearance, and in the immunity so established the outstanding effect is the increased activity of the body fluids favoring phagocytosis, although the existence of a slight degree of residual and purely cellular immunity cannot be excluded. Because leucocytes could be considerably decreased in number without in-

terfering with the capacity of the animal to dispose of organisms introduced into the blood, taken in connection with the specific action of serum on the cocci, Wright laid particular stress on the importance of humoral immune elements in bringing about the destruction of invading pneumococci.

That circulating antibodies and not leucocytes play the dominant part in recovery from pneumococcal infection was also the contention of Robertson, Woo, Cheer, and King (1928).[1152] The blood of cats and rabbits surviving experimental pneumococcal infection possessed the ability to promote the destruction of highly virulent pneumococci in rabbit serum-leucocyte mixtures, which mixtures in themselves had no growth-inhibitory action. The bactericidal action of the serum was associated with a marked increase in acquired resistance to infection. In cats, which were studied in greater detail, the pneumococcidal promoting power of the serum as well as the opsonic, agglutinative, and mouse-protective properties, which were found to be type-specific, became demonstrable at the time of recovery, and their appearance in the serum always marked the termination of blood invasion. The animals succumbing to infection failed to develop detectable immune properties in the serum and showed persistent bacteriemia. According to the authors, the degree of leucocytosis had no constant relation to the outcome of the disease.

In two communications, Terrell (1930)[1385-6] presented the results of experiments carried out to determine the changes in humoral immunity occurring during the early stages of experimental pneumococcal infection. Using the technique of Robertson and Sia[1144-5] to determine circulating antibodies, the author, after infecting normal cats and dogs with virulent cultures of Type I and II pneumococci, found that in a generalized and overwhelming infection accompanied by early blood invasion, there was a prompt and rapid decrease in the concentration of native humoral immune bodies, which frequently disappeared entirely by the time of death. Animals surviving a moderately severe, generalized infection

showed a similar early diminution of humoral immune substances, but with the onset of recovery the concentration of the immune bodies again rose. When the infection was localized, as in the case of true lobar pneumonia, the presence of humoral antibodies in quantity could be demonstrated in the blood throughout the course of an infection terminating in death.

Other evidence indicating the participation of leucocytosis in resistance to pneumococcal infections is to be found in the communication of Schattenberg and Harris (1932).[1222] The plan of the experiments was to induce leucocytosis in white mice by preliminary injections of killed cultures of typhoid bacilli or staphylococci, of detoxified suspensions of devitalized pneumococci, and of sterile milk. Six to eight hours later the animals were inoculated intraperitoneally with lethal doses of pneumococci of Types I, II, and III so measured that death of the mice could be controlled to take place at periods varying from three to twenty-four hours. The animals thus treated failed to show that stimulation of leucocytosis had any effect in preventing the development of pneumococcal peritonitis.

NORMAL TROPINS OR OPSONINS

The ability so to sensitize pneumococci that they become attractive to leucocytes and susceptible to phagocytic lysis is a character of some normal as well as of immune serums. Rosenow[1160-1] disclosed the property in normal human serum and found opsonins for streptococci, staphylococci, and tubercle bacilli, in addition to those for pneumococci.

Ungermann (1911)[1434] was unable to discover any phagocytosis of virulent pneumococci in the serum of normal mice and rabbits. Avirulent strains were readily phagocyted, and the degree of the action appeared to parallel the resistance of the particular species of animal whose serum was used. The conclusion was reached that normal resistance depended upon the phagocytic power of fresh normal serum, at least in the cases studied, and that the serum must

develop this power *in vitro* in the presence of leucocytes. Woo[1541] later (1926) found, in testing the pneumococcidal activity of normal serum-leucocyte mixtures, that virulence of the cultures and the age of the test rabbit influenced the outcome of the test. Avirulent strains were readily destroyed in a mixture of normal rabbit serum and leucocytes, whereas virulent cultures resisted the destructive action of the mixture. The absence of pneumococcidal properties in the blood of very young rabbits agreed with the extreme susceptibility to pneumococcal infection of immature animals of the species.

Strouse,[1346] by both *in vitro* and *in vivo* tests, demonstrated the same quality in the peritoneal fluid of pigeons. Wright[1547] noted the rapid disappearance of virulent pneumococci injected directly into the blood stream of rabbits and the still more rapid removal of avirulent forms. Specific immunization enhanced the activity of body fluids favoring phagocytosis. Robertson and Sia,[1144] and later Sia,[1268] not only proved the existence of normal opsonins for pneumococci in the blood of cats, dogs, sheep, and pigs but, by absorption experiments, exhausted the serum of opsoninizing substance for the strain used as absorbent without removing opsonins for organisms of other types. The results suggest that there are separate type-specific opsonins for pneumococci in the serum of animals naturally resistant to pneumococcal infection.

In 1933, Ward and Enders[1484] published a communication, based on experiments with Pneumococcus, dealing with a serological analysis of the opsonic or tropic action of normal and immune serum. The authors studied the action of the anticarbohydrate substance in normal serum as an opsonic or tropic agent, and also the properties of normal and immune serum which promote phagocytosis after the anticarbohydrate substance or antibody had been removed from the serum by appropriate amounts of type-specific polysaccharide. The authors analyzed the mechanism of the phagocytosis of Pneumococcus as follows: In normal, unheated human serum virulent pneumococci may be prepared for phago-

cytosis by two separate antibodies acting in conjunction with complement. One of these is the type-specific anticarbohydrate antibody reacting with the carbohydrate fraction of Pneumococcus; the other is probably also a type-specific antibody, but quite distinct from the former and, therefore, reacting with a different antigenic constituent of the bacterium. In normal human serum heated to 56° these two antibodies may, after prolonged contact with the organism, promote phagocytosis of Pneumococcus without the adjuvant action of complement. The two antibodies are equally effective in the phagocytosis of twenty-four-hour cultures by normal blood, but the anticarbohydrate antibody tends to predominate as the pneumococci approach the state in which they exist in the animal body. The anticarbohydrate antibody was the only one in immune serum which could be demonstrated to induce phagocytosis. It was active by itself, but complement enhanced its effect.

To Ward and Enders it seemed that a single well-defined antibody—the anticarbohydrate antibody—might be responsible for the phagocytic action of unheated normal serum, of heated normal serum, inactivated immune serum, and immune serum activated by complement. The facts appeared to invalidate the division proposed by Neufeld of the phagocytic antibodies into bacteriotropins—antibodies, the phagocytic titer of which is not raised by the addition of complement—and opsonic antibodies—antibodies, comparable to lysins, which are only active in the presence of complement. Complement alone was found to be incapable of inducing phagocytosis of Pneumococcus and, therefore, may act merely as a catalyst in increasing the velocity of the phagocytic process. On the basis of their observations, Ward and Enders proposed that the term "tropin" be discarded, since it was misleading and unnecessary, and that the term "opsonin" be retained to denote any heat-stable antibody which prepares bacteria for phagocytosis. Contrary to current usage the latter term would not suggest a combination of antibody with complement.

ANTIOPSONINS

The existence of substances in Pneumococcus inimical to leuco-
cytic ingestion became apparent to Rosenow (1907)[1161] who, by
saline extraction or by autolysis of the cocci, obtained a substance
or substances that inhibited opsonic action. It was found further
that avirulent strains could absorb the inhibiting substance and
then become resistant to phagocytosis, while after similar extrac-
tion virulent organisms acquired the capacity to absorb opsonin
and became vulnerable to the destructive action of the white blood
cells. The presence of substances in pneumococci capable of inhib-
iting phagocytosis was demonstrated by Tchistovitch and Youre-
vitch[1383] in 1908. When saline suspensions of washed, virulent
pneumococci were added to serum-leucocyte mixtures, marked
phagocytosis resulted, but when a small amount of the washings
from the original culture was added to the combination the cocci
were no longer ingested by the leucocytes. The inhibiting sub-
stances were found only in cultures of virulent strains, they were
specific for the bacterial species and were thermostable. For such
substances Tchistovitch proposed the name "Antiphagins."

Pritchett[1110] endeavored to develop antiopsonins in rabbits by
injecting them with antipneumococcic horse serum, but could ob-
tain no evidence of the formation of substances antagonistic to the
action of immune serum. On the contrary, the serum of rabbits in-
jected with antipneumococcic horse serum for Type I, II, or III
pneumococci, when combined with antipneumococcic serum for
Types I and II, increased opsonization of organisms of Types I
and II but in this respect never affected strains of Type III.

Confirmation of the existence of antiopsonic principles in viru-
lent pneumococci is to be found in the experiments of Wadsworth
and Sickles,[1475] and of Sickles (1927).[1277] Intracellular substances
released by sodium oleate solution of the organisms, or present in
the sterile filtrates of broth cultures, prevented phagocytosis of
pneumococci in immune-serum and leucocyte mixtures. There was
some evidence of type-specificity in the action of the pneumococcal

derivatives, since an extract of Type I Pneumococcus failed to prevent phagocytosis of Type III organisms in the presence of Type III antiserum, and vice versa. The inhibiting substance affected the organisms and not the leucocytes. Furthermore, the active principle could be absorbed from extracts by combination with specific immune serum.

Yamamoto (1929)[1557-8] studied the effect of both unheated and heated culture filtrates of pneumococci on spontaneous phagocytosis *in vivo* in the rabbit. The "Impedin," as the author called the inhibitory principle, was without action on the number of migrated leucocytes. The injection of heated filtrates affected phagocytosis in inverse ratio to the duration of previous exposure of the filtrate to heat, the antiopsonic property being completely destroyed after thirty to sixty minutes at boiling temperature.

The action of the specific capsular polysaccharide, so conspicuous in the inhibition of other serological reactions between Pneumococcus and immune serum, is manifested in its antagonistic effect on opsonins, and is referable to its function of combining with and precipitating the antibodies from serum. In studies on growth inhibition, Sia (1926),[1267] by means of normal rabbit or cat serum-leucocyte mixtures, learnt that the presence of a very small amount of purified soluble specific substance from pneumococci of both Types I and II markedly altered the conditions in the mixture so that even a small number of avirulent pneumococci were enabled to grow in the presence of serum and leucocytes of animals which ordinarily possess the power to destroy the organisms in relatively large numbers. The action of the capsular carbohydrate was type-specific, and the same neutralizing effect on the growth-inhibitory or pneumococcidal power of normal serum-leucocyte mixtures was exhibited by broth filtrates of cultures of young pneumococci.

Ward[1480-1] confirmed Sia's observations on the specific antiphagocytic action on blood of soluble specific substance, and found the action more marked in the case of Type III than Type I pneu-

mococci. In *in vitro* phagocytic experiments with human blood, antipneumococcic serum, capsular polysaccharide, and living, virulent pneumococci, there was a zone of definite phagocytic inhibition when a strong antiserum was used. Upon dilution of the serum there appeared a zone in which phagocytosis was effective. Ward suggested that the inhibition was caused by the specific precipitate formed by the combination of soluble specific substance and precipitin, which interfered, perhaps mechanically, with the ingestion of the pneumococci by leucocytes.

In later reports, Ward[1482-3] described a type-specific substance with a powerful antibacterial action present in the filtrate of a five-day broth culture of Type III Pneumococcus. A similar substance was also demonstrated in the filtrate of a lung obtained at necropsy from a patient dying of lobar pneumonia caused by a Type III organism. Ward employed a method devised by himself for estimating the pneumococcidal action of whole blood, and found that if the precipitinogen content of the broth filtrate and the amount of soluble specific carbohydrate of Type III Pneumococcus were taken as a basis of comparison, it required approximately one thousand times as much antiserum to neutralize the antagonistic action of the broth filtrate as was necessary to neutralize the specific carbohydrate. Ward also found that a specimen of blood from a patient convalescing from Type III lobar pneumonia, though comparatively weak in anticarbohydrate antibody (precipitin), was better able to neutralize the broth filtrate and the lung filtrate than a corresponding mixture of normal blood and specific antipneumococcic serum.

Enders and Wu (1934)[362] more recently reported that the opsonic titer of normal serum could be practically abolished by the addition of the A carbohydrate. In immune serum, the A substance brought about a quantitatively greater reduction in opsonic activity than did its derivatives, but the authors were never successful in demonstrating complete inhibition of phagocytic action by the method of absorption of antibody. Enders and Wu also

showed that the A carbohydrate was far more effective as an anti-bactericidal—that is, antiopsonic—agent than deacetylated derivatives of the capsular polysaccharide.

OPSONINS IN PNEUMONIA

Among others, Rosenow (1906)[1160] announced that the content of opsonins for Pneumococcus appeared to be less in the serum of patients succumbing to pneumonia than it was in normal human serum or in serum of patients during or after crisis. Using the Neufeld technique, Strouse (1911)[1346] could demonstrate no phagocytosis of virulent pneumococci by serum obtained from pneumonia patients after crisis, but by sensitizing the organisms with convalescent serum previous to the test, phagocytosis was observed in about one-fourth of the cases. Eggers (1912),[349] employing the plate method of cultivation, noted an increase in the pneumococcidal power of the blood of pneumonia patients at or near the time of crisis, and while he demonstrated that the action was due to phagocytosis, Eggers presented no detailed experimental evidence to prove whether the action of serum was exerted on the cocci or on the leucocytes. Clough (1913)[240] observed that the serum of approximately one-half the pneumonia patients obtained after crisis or lysis exhibited definite phagocytic activity, and that the reaction was strictly limited, under the conditions of the test, to the homologous strain of Pneumococcus isolated from the patient whose serum was being examined. Clough concluded that, since the active substances resisted heating at 56° and persisted in the serum *in vitro* for a considerable period of time, the substances must be tropins and, since phagocytic activity of the serum appeared to parallel closely the protective power for mice in incidence, time of appearance, and strain specificity, the protective action of serum was directly due to its ability to promote phagocytosis. In a second report, Clough (1919)[242] confirmed his earlier observations and added that while in 85 per cent of cases of acute lobar pneumonia the serum showed positive phagocytic activity

after crisis or lysis, it lacked the property in the great majority of instances where the patient had died of the disease. The height of bacteriotropic activity of the serum of pneumonia patients was found by Adler (1923)[3] to occur at the time of crisis.

THE MECHANISM OF PHAGOCYTOSIS

The factors and processes involved in the response of rabbits to intravascular injection of pneumococci were investigated by Kitagawa (1915).[717] When the living organisms were injected directly into the blood stream of rabbits there was an immediate, initial drop in the count of circulating cocci, followed by either a slow increase or decrease in number depending upon the dosage and virulence of the injected culture. When similar injections were made into actively immunized rabbits, the cocci disappeared with great rapidity from the circulation, the blood usually becoming sterile within ten minutes after the injection. Kitagawa concluded that the speedy disappearance of the injected pneumococci from the blood stream of actively immunized rabbits was not due to destruction of the organisms by plasma or leucocytes, but rather to their mechanical removal or destruction by the fixed tissues. Kline and Meltzer (1915)[727] also noted that pneumococci promptly vanished from the pneumonic lung of dogs infected by intrabronchial insufflation after previous repeated intravenous injections of pneumococci.

The same phenomenon was studied by Winternitz and Kline (1915).[1522] In normal rabbits the reaction to inoculation was immediate and the ultimate result was bacteriemia and death; in passively immunized animals the organisms rapidly disappeared from the blood and recovery took place; in rabbits previously rendered aplastic with benzol and then injected with specific immune serum, there was an immediate effect on the injected pneumococci, but bacteriemia recurred and death ensued; in actively immunized, aplastic rabbits, the initial reaction was the same as in the passively immunized, aplastic rabbits, but the actively immunized,

aplastic rabbits recovered. Winternitz and Kline accordingly concluded that the immunological response was dependent upon at least three factors, namely, immune bodies, white blood cells, and a third factor relying for its existence on the presence of white blood cells at the time of inoculation with Pneumococcus. The humoral immune bodies acted in causing the immediate disappearance of the injected organisms from the circulation, but the third factor, originating in the leucocytes, appeared to be essential to the recovery of the infected animal.

The importance of elements peculiar to immune serum for the protection of mice was shown by Tilgren (1915)[1401-2] who, by adding immune sheep serum and sheep leucocytes to an infective dose of pneumococci, protected rabbits against infection. In mice, the immune serum afforded protection but the addition of heterologous leucocytes resulted in no increase in the protective power of the serum.

More detailed information regarding the tissues concerned in resistance to pneumococcal infections is to be found in the communication of Permar (1923),[1082] who believed that the mononuclear phagocytic cells appearing in the exudate in acute experimental pneumonia in the rabbit were of vascular endothelial origin. Other cells of visceral origin possibly contributed in some degree to the total number of phagocytes in the exudate. Dust cells of similar origin already present in the alveoli also might act as phagocytes in inflammatory reactions, while interstitial dust cells could become reactivated and through liberation by inflammatory edema were enabled to appear in the exudate. The entire group of cells, therefore, including the newly produced phagocytes and the dust cells, may take part in the reaction to the invading pneumococci.

Singer and Adler (1924),[1290] after studying the response in rabbits to infection with Type III Pneumococcus, believed that the defensive factors resided in the reticulo-endothelial system, and that the histocytes of bone marrow, the capillary endothelia of the parenchymatous organs, the endothelium of serous cavities, and

the biologically comparable alveolar epithelia, as well as the fixed tissue cells with phagocytic properties underlying them, must have experienced in the immune animal a specific alteration in the sense of acquiring the power to ingest pneumococci and to retain and eventually to kill the organisms. To Singer and Adler, leucocytic phagocytosis merely exerted a secondary chemotactic action, but had nothing to do with specific immunity toward a given organism. In a second communication in the same year, Singer and Adler[1291] reported analogous results obtained in experiments of the same nature with pneumococci of Types I and II. The authors maintained that in passive immunity the cellular system played the main part, the action of the serum being purely a bacteriotropic one. The phenomenon was definitely type-specific and the elements of the reaction were located in the reticulo-endothelial tissues, since functional blockade of that system caused a disappearance of the manifestations of immunity.

Tudoranu[1427] disagreed (1926) with the theory of Singer and Adler that immunity toward Type III Pneumococcus was devoid of specific humoral elements and quoted the latter's own experiments to refute the claim. Although in passively immunized rabbits injected with Type III cultures the organisms for a time multiplied in the blood, the animals eventually recovered, whereas normal rabbits similarly inoculated succumbed to infection. Tudoranu, in support of the contention, cited his success in passively immunizing rabbits by the subcutaneous, intraperitoneal, and intravenous injection of specific immune serum, and suggested that the successful result was merely a question of dosage of serum. The author concluded that the action of the serum was to neutralize the aggressins, thus permitting the production of an exudate rich in leucocytes, which modified the cocci in such a way as to render phagocytosis possible.

In the interval between the publication of the papers of Singer and Adler and of Tudoranu, Neufeld and Meyer (1924)[995] reported the results of a study of the origin of antipneumococcal

immunity. According to the latter authors, actively immunized mice manifested the same specific phagocytosis as those passively immunized, but scarcely ever had demonstrable protective bodies in the blood. However, after intravenous injection of manganese salts, the protective substances appeared in the blood in large amounts. Neufeld and Meyer concluded that the active immunity depended entirely upon antibodies, that the antibodies were formed in the reticulo-endothelium and that, in a broader sense, the cells of the *Gefässbindegewebsapparat* were the sole source of antibodies.

The ability of macrophages to dispose of pathogenic bacteria was described by Nakahara (1925).[941] The method of demonstrating the action of these cells was the injection of olive oil into the peritoneal cavity of mice. The injection was followed at first by the appearance of polymorphonuclear leucocytes, which were largely replaced by mononuclear cells. When pneumococci were injected into mice during the macrophagic reaction, the organisms were destroyed more rapidly than was the case in normal animals, and the mice survived multiples of the minimal infecting dose. The macrophages were observed to phagocyte the injected cocci actively.

Meyer (1926),[896] after blocking the reticulo-endothelial system by the intravenous injection of ferric saccharate, India ink, trypan blue, and other dyes, along with the removal of the spleen, was unable, as a rule, to immunize mice by the injection of killed pneumococci of Type I. When active immunization preceded the removal of the spleen and the injection of the blocking agents, the immune condition of the animals was sometimes altered. Meyer was not able to substantiate the earlier observation of Neufeld and Meyer on the necessity of stimulating the endothelia by manganese, since protective antibodies were found to be present in the blood of the majority of mice immunized with Type I pneumococci. Inasmuch as splenectomized mice in which the reticulo-endothelial system had been blocked easily acquired passive protection after

the injection of specific immune serum, Meyer argued that the effect of these measures could not be due to a decrease in the phagocytic activity of the reticulo-endothelial cells, but rather to an inhibition of antibody formation in the cells and that active as well as passive immunity depended upon specific antibodies circulating in the blood and present in the cells. The contribution of the reticulo-endothelial system to the operation of the immune mechanism was believed by Wright (1927)[1547] to be of relatively slight importance. Although he acknowledged that the possibility of a residual cellular immunity could not be overlooked, Wright considered circulating antibodies to be the prime factor in immunity to Pneumococcus.

The relationship of blood-free splenic cells of normal and immunized rabbits was investigated by Loewenthal and Micseh (1929).[822] Using the technique of tissue culture devised by the first-named author, it was found that the macrophages of the spleen of normal rabbits phagocyted avirulent pneumococci only in the presence of fresh, normal serum, and phagocyted virulent organisms only in the presence of specific immune serum. Macrophages of the spleen of immunized rabbits phagocyted both avirulent and virulent pneumococci without the addition of either normal or immune serum, when the tissue fragments were not transplanted into a fresh medium. However, when bits of spleen tissue of immunized rabbits were transplanted into fresh media during tissue culture, the macrophages behaved similarly to those of normal animals. Loewenthal and Micseh believed that the results pointed to a close relation between the mechanisms of humoral and cellular immunity.

In 1932 and 1933, Kritschewski, Rubinstein, and Heronimus[758-9] confirmed the results of Meyer on the inability of antipneumococcic serum to protect mice that had been splenectomized and had suffered artificial impairment of function of the reticulo-endothelial system. The great majority of mice so treated succumbed to intraperitoneal inoculation with Type I Pneumococcus.

Protective Antibodies

To resolve the protective action of antipneumococcic serum into the causal elements participating in the phenomenon is a difficult task. There is the innate defense mechanism of certain animal species; the functional ability of various animals to respond to antigenic stimuli, as manifested by the elaboration of antibodies of different properties; the interaction of tropins or opsonins, cocci, and white blood corpuscles; and the natural or the artificially enhanced avidity of fixed tissue cells for pneumococci. As in other aspects of antipneumococcal immunity, the communications of the Klemperers (1891)[724-5] were prophetic in suggesting that the serum of animals after being injected with derivatives of Pneumococcus developed the ability to render the cocci susceptible to the action of blood cells and body cells and, therefore, harmless to the injected animal. The authors erroneously ascribed the protective action of the serum to an antitoxic effect, but the assumption does not invalidate the observation. Eyre and Washbourn (1898),[374] by injecting mice with mixtures of varying amounts of antipneumococcic horse serum and virulent cultures of pneumococci, were the first workers to demonstrate quantitatively the protective power of specific immune serum. The authors later applied the method to the standardization of therapeutic serum and, in doing so, discovered that the method could be employed to distinguish between different varieties of the organism.*

Neufeld and Haendel (1910),[991] using the protection test for evaluating the strength of immune serum, confirmed the observations of Eyre and Washbourn on the existence of types of pneumococci with different serological affinities and thus established the protection test as a means for the classification of pneumococci into different serological types. Neufeld and Haendel at first employed 0.2 cubic centimeter of immune serum, which they in-

* Wassermann (1899)[1491] sought to locate the origin of protective substances, and from his experiments concluded that the antibodies apparently were formed in the bone marrow, and that the lymph nodes, thymus, and spleen merely served as reservoirs.

jected intraperitoneally into mice weighing 18 to 20 grams two to three hours before giving an intraperitoneal inoculation of varying amounts of virulent broth cultures. Later, when more potent serum became available, falling amounts of serum were injected into mice just previous to inoculation with one or more multiples of the minimal infecting dose. Ungermann and Kandiba[1436] applied the Neufeld and Haendel technique to a study of the quantitative relations between the protective strength of immune serum and the amount of culture used, and discovered that the action of the serum failed to follow the law of multiple proportions. When the tests were performed on rabbits, there appeared to be a definite relation between the volume of serum and the weight of the animal, called by Ungermann and Kandiba (1912) the *Schwellenwert* or threshold value. Above this value serum protected against many multiples of the minimal lethal dose of culture, but below the threshold the serum had little if any action. In their experience, Neufeld and Haendel (1912)[993] found that, in order to ensure protection with serum, it was necessary to cross the hypothetical threshold and to administer large volumes of serum.

SPECIFICITY

Some claims have been advanced concerning the lack of type-specificity in the protective mechanism. For example, Yoshioka (1923)[1561] reported that mice that had survived an intraperitoneal inoculation of pneumococci were protected against an intraperitoneal injection three or four days later of cultures of heterologous type. Furthermore, mice similarly treated were able to withstand inoculation with streptococci, while large doses of killed staphylococci produced definite protection against pneumococci. Yoshioka also noted a certain degree of cross-protection in guinea pigs and rabbits after pneumococcal infection. Kolchin and Gross (1924)[739] observed cross-protection for Type III strains in a sample of Felton's monovalent Type I antipneumococcic serum. Mono-

valent rabbit antiserum prepared with pneumococci of Types II and III also exhibited cross-protection between Type III serum and Type I organisms. Despite the heterologous action observed in the protection test, all the samples of immune rabbit serum tested were found to be type-specific in agglutinin content.

From the data presented in the communications of Yoshioka and of Kolchin and Gross it is difficult properly to interpret the results. However, against the claims of the occurrence of cross-protection with monovalent immune serum there is a mass of evidence attesting the specificity of the reaction when serum is prepared from carefully authenticated strains of Pneumococcus and when similarly identified strains are used for testing the protective action of immune serum. The mouse protection test, when properly executed, therefore, remains the final criterion for establishing the type-identity of pneumococcal strains.

RELATION TO OTHER ANTIBODIES

A definite relation exists between the amount of protective antibody in a given serum and its content of agglutinins and precipitins. Among the many references which might be cited, the work of Heidelberger, Sia, and Kendall (1930)[630] and the study of Felton (1931)[407] are chosen as illustrative. The former authors determined a close parallelism between the specifically precipitable protein and the number of mouse-protective units in a wide variety of Type I antipneumococcic serums. So constant were the results yielded by the simple method for determining the amount of precipitable protein in immune serum that the reaction as described in the section of the present chapter dealing with precipitins could be relied upon to measure the protective strength of the serum. Sabin (1931)[1204] was unable to precipitate the totality of protective antibody from antipneumococcic serum by the addition of homologous soluble specific substance and hence assumed the existence of a protective substance distinct from the anticarbohydrate antibody. However, Felton[407] found in samples of Type I anti-

pneumococcic horse serum a close correlation between protection, precipitin, and agglutination titer for Type I Pneumococcus.* Similarly, Barnes, Clarke, and Wight (1936),[82] using a modified precipitin test, found that the correlation between precipitin and protective antibody content was sufficiently close to warrant the substitution of the precipitin test for the animal test in titration of the protective value of antipneumococcic serum.*

ADDITIONAL DATA

Kyes (1911)[765] employed domestic fowls for the preparation of antipneumococcic serum, giving the birds repeated inoculations of large doses of virulent pneumococci. Kyes concluded that by this method it was possible to obtain serum possessing distinct protective action against pneumococci within certain hosts. In 1920, Wadsworth,[1460] after injecting a single dose of standardized pneumococcal vaccines of Types I, II, and III into mice, found that it was possible with Type I vaccine to produce protection against Type I cultures but not against Type II or III strains. In the case of Type II vaccine there appeared to be protection exclusively against the homologous organism while, when Type III vaccine was administered, there was no protection against any of the three types. Cecil and Blake,[206] after injecting monkeys with saline suspensions of heat-killed virulent Type I pneumococci, could demonstrate protective antibodies in the serum although the animals eventually succumbed to intratracheal inoculation with the same living strains. Later, Cecil with Steffen,[213] by the intravenous and subcutaneous injection of larger doses of pneumococcal vaccine, succeeded in completely protecting monkeys against infection and made the seemingly paradoxical observation that protective substances might or might not be present in the serum of the immune monkeys.

The same authors[214] reported (1925) a similar condition in monkeys immunized by intratracheal vaccination, and decided that

* See page 378 of this chapter.

the immunity so produced was probably cellular in nature. Barach,[74] employing mice and rabbits, was able by the injection of heat-killed pneumococci of Types I and II, and of sterile culture filtrates of the organisms, to demonstrate the presence of protective antibodies in the serum of the animals three days after injection. The degree of immunity produced was greater in the case of Type I than of Type II vaccine, and Barach concluded that immunity was due to the elaboration of protective antibodies. Barach (1931)[76] obtained analogous results after injecting similar vaccines prepared from heterologous strains of pneumococci into patients suffering from lobar pneumonia. The average time of appearance of protective antibodies was between five and six days after intravenous and intradermal administration of the killed pneumococci. Similar effects followed the intradermal injection of protein-free, type-specific polysaccharide from pneumococci of Types I, II, and III into normal individuals, as reported by Finland and Sutliff (1932).[448]

In the year previous, Avery and Goebel[45] had demonstrated that the appearance of type-specific protective antibodies followed the injection into rabbits of the capsular polysaccharide of Type III Pneumococcus conjugated with horse-serum globulin, and that it was the polysaccharide which determined the type-specificity of the ensuing protective antibodies. Avery and Goebel[46] then (1933) proved that the acetyl polysaccharide could induce the development of the specific protective substance in animals. Confirmation of the formation of type-specific protective antibodies after injection of the capsular polysaccharide was forthcoming from the study of Francis (1934)[474] on the immunizing effect of the intradermal injection of small amounts of pneumococcal carbohydrate into man.

CHEMICAL NATURE OF PROTECTIVE ANTIBODIES

The work of Avery (1915)[32] yielded the first information concerning the particular protein element in antipneumococcic serum

with which the specific protective antibodies are associated, and furnished the foundation for the future development of methods for the isolation and concentration of the protective substance. From the serum of horses immunized with pneumococci of Types I and II over a period of one to two years, Avery was able, by adding ammonium sulfate to 38 to 42 per cent saturation, to precipitate completely the immune bodies. The protein fraction holding the protective antibodies did not correspond to the euglobulin of the serum since the fraction was not rendered insoluble by one-third saturation with ammonium sulfate or by complete saturation with sodium chloride. In 1924, Felton,[396] by the use of water acidulated with tartaric acid, separated the bulk of protective substance from antipneumococcic serum of Types I, II, and III. The precipitate appeared to be composed largely of globulin, which in the state of purity obtained at the time by Felton had an isoelectric zone between 6.6 and 7.5.

Banzhaf,[70] by Felton's method of dialysis, separated approximately 90 per cent of the protective bodies from the fraction of globulins precipitable between the limits of 30 and 50 per cent saturation with ammonium sulfate. In further studies, Felton[397-400, 403] found that the water-insoluble globulin retained its protective power after repeated purification, and that the globulin could be largely freed from accompanying serum proteins through precipitation with appropriate concentrations of sodium sulfate and by ethyl alcohol.[408] The possible protein nature of the immune substance was suggested by the fact that its protective value was diminished by the digestive action of pepsin, trypsin, pancreatin, and papain (Felton and Kauffmann, 1927).[426]

Goodner,[528] by the addition of appropriate amounts of water, was able to precipitate the protective antibody as well as agglutinins from antipneumococcic serum. The dilution of serum, after preliminary tests to determine the proper amount of water to be added, was carried out at a temperature of approximately 4°. After carrying the precipitate through a refining process, the re-

sulting solution was said to contain 14,000 Felton protective units
per cubic centimeter.

The question arose whether the protective principle against
Pneumococcus in specific immune serum is actually a protein of
the globulin type representing merely an increase in the native se-
rum globulin, whether it is a new protein substance associated with
but chemically distinct from the natural globulin of serum, or
whether it is a non-protein substance sharing some of the physical
and chemical properties of serum pseudoglobulin. From time to time
various answers have been proposed. The work of Avery, of Banz-
haf, and of Felton indicated that the protective antibody is inti-
mately associated with the pseudoglobulin fraction of immune se-
rum, but the results do not necessarily imply that the antibody is
the natural serum globulin. For example, the experiments of Fel-
ton and Kauffmann showed that the ratio of protein to protective
units can be reduced by selective salting out with ammonium sul-
fate. A difference between immune and native serum globulin is the
more alkaline isoelectric point of water-insoluble globulin contain-
ing protective antibody, as shown by Felton and by Reiner and
Reiner.[1133] The unusual alkaline isoelectric point of the purified
immune proteins was considered by Chow and Goebel[226] to be its
most characteristic property, and the authors believed that this
property might be attributed to the relatively high ratio of amino
to carboxyl groups present in the protein molecule.

Chow and Goebel by repeatedly precipitating pneumococcal an-
tibody with ammonium sulfate were able to rid the specific pseudo-
globulin from much inert protein material, and then by means of
potassium acid phthalate they succeeded in effecting a further
purification of the pseudoglobulin antibody. Although Huntoon[666]
believed that the specific protective antibody separated by him
from pneumococcal antigen-antibody precipitates was non-protein
in nature, Chow and Goebel were of the opinion that the circulat-
ing antibodies are in reality modified serum globulins. Confirma-
tion of the latter hypothesis has come from the work of Heidel-

berger and Kendall,[625] who, by the use of strong salt concentration and proper adjustment of the hydrogen ion concentration well on the alkaline side, obtained from pneumococcal polysaccharide-homologous antiserum precipitates a protein substance of which 93 per cent of the total nitrogen was in the form of immune body nitrogen.

Heidelberger and Kendall took the precaution of removing from their source material—unconcentrated Type I, II, and III anti-pneumococcic serum—somatic protein antibodies and somatic car-bohydrate (Fraction C) antibodies, thus reducing the antibody content of the serum to antipolysaccharide antibodies. By employ-ing unconcentrated serum without chemical treatment, any pos-sible denaturation of the antibody was avoided and the antibody dissociated from the specific precipitate was considered as being presumably free from artificial concomitants.

By dissociating antibody from similar antigen-antibody precipi-tates and then by purifying the antibody by dialysis with subse-quent precipitation at its isoelectric point, Chow and Wu[227] suc-ceeded in obtaining a protein preparation of high precipitating activity, which they considered as representing an immunologically pure protein.

The work of Wyckoff[1555] and of Heidelberger, Pedersen, and Tiselius[629] further attests the protein nature of pneumococcal an-tibody and points to a distinction between specific immune globu-lin and the globulin of normal horse serum. However, there still remain discrepancies to be explained. None of the antibody prepa-rations so far obtained can be regarded as pure in the strict sense of the word from either a chemical or an immunological viewpoint. As Chow and Goebel remarked, no end-product ever attains a value of 100 per cent of type-specific precipitable protein. Yet the evi-dence that has accumulated in the past few years makes it difficult to escape the conviction that pneumococcal antibody is a protein, undoubtedly of the pseudoglobulin type, and that it is a chemical entity distinct from native serum pseudoglobulin.

Further information concerning the intimacy existing between pneumococcal antibody and immune serum globulin has come from analyses of normal and antipneumococcic serum by means of the ultracentrifuge of Svedberg[1369] and a modified machine described by Biscoe, Pickels, and Wyckoff (1936).[119] Employing the latter apparatus, Biscoe, Herčík, and Wyckoff[118] determined that in concentrates of Type I antibodies the proteins consist mainly of molecules with a sedimentation constant of about

$$16 \times 10^{-13} \text{ cm. sec}^{-1} \text{ dynes}^{-1}.$$

This finding, taken with the presence of similar molecules in unconcentrated antipneumococcic horse serum and their complete or almost complete absence from normal serum indicates that these molecules are the real bearers of antibody activity. Further support of the view is supplied by the observation of Heidelberger, Pedersen, and Tiselius[629] that after ultracentrifugation the specific antibody from serum from a horse immunized with Type I polysaccharide and a sample of concentrated Type I antipneumococcic serum (Felton) showed homogeneous sedimentation, with the following sedimentation constant:

$$s_{20} = 17.2 \times 10^{-13}$$

The sedimentation constant of normal globulin from mammalian serum is:

$$s_{20} = \text{about } 7 \times 10^{-13}$$

By applying the method to concentrated preparations of Type I antipneumococcic serum, Wyckoff[1555] found that the only protein molecules in the bottom layers of the fluid were those with

$$s = 16 \times 10^{-13}$$

Small amounts of light molecules, which in each concentrate accounted for roughly 15 per cent of the total protein content, were those of the principal serum globulin with

$$s = 7 \times 10^{-13}$$

The experiments strongly support the idea that the Type I antibody is associated with the molecule having

$$s = 16 \times 10^{-13}$$

Furthermore, Biscoe, Pickels, and Wyckoff obtained the same measurement for molecules of antibodies against other types of pneumococci. Speculating on the relation between pneumococcal antibody and specific immune protein, Wyckoff concluded: "Since this is a molecular species that appears in appreciable quantities in horse serum only when it has become antipneumococcic, two possibilities suggest themselves. It is conceivable that during immunization this globulin is made or freed in excess in order that there may be plenty of it present to fix all the antibody activity that may develop . . . and the alternate, that the globulin is the antibody." The results of investigations of this nature are highly suggestive, and the method promises to yield valuable information concerning this abstruse but important question.

Differences in the nature of the protective antibody in antipneumococcic rabbit serum and in that in antipneumococcic horse serum have been demonstrated by Chow.[224] In horse serum, the antibody in the pseudoglobulin fraction soluble at pH 5.5 was completely precipitated when the reaction of the solution was adjusted to pH 7.6. From the corresponding fraction of the immune rabbit serum no trace of precipitate was obtained. Furthermore, in the case of immune horse serum, the main portion of the antibody was concentrated in the pseudoglobulin fraction, whereas the opposite was found to be true with the immune rabbit serum, that is, the euglobulin fraction apparently contained the major portion of the antibody.*

Another difference in the antibodies present in antipneumococcic rabbit and horse serum lies in the size of the molecule of the two substances. In experiments involving the use of the ultracentri-

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* A difference in the phosphatid molecules linked with the globulin in the case of antibodies from the horse and rabbit has been mentioned in the footnote on page 381 of this chapter.

fuge, Heidelberger, Pedersen, and Tiselius observed that in the case of immune rabbit serum the specific antibody was produced from the principal globulin component, while in the horse the anti-carbohydrate for Type I Pneumococcus is developed from an otherwise minor component. The isoelectric point of the protein representing the pneumococcal antibody of the rabbit was determined as approximately pH 6.6, whereas the isoelectric point of the immune protein from horse serum was as acid as pH 4.8. That the molecule of the protective antibodies as elaborated by the two animal species is of different size is also evident from the work of Goodner, Horsfall, and Bauer.[589] When Type I antipneumococcic rabbit serum was filtered through an ultrafilter of the type described by Bauer and Hughes,[90] no appreciable specifically precipitable protein passed through a membrane with an average pore diameter of one millimicron. A 13.8 millimicron filtrate contained 11.9 per cent of the total specifically precipitable protein. Slightly greater amounts were recovered as the pore sizes were increased up to 73 millimicrons, at which point the curve rose sharply until at 102.5 millimicrons the filtrate contained 86.6 per cent of the total antibody. With antipneumococcic horse serum, the smallest pore permitting the passage of antibody was 45.2 millimicrons. Between 73 and 102.5 millimicrons the curve rose steeply until at the latter porosity 76.7 per cent of the antibody was recovered. With a concentrated preparation of Type I anti-pneumococcic horse serum the end-point was relatively sharp, no antibody being recovered at 150.4 millimicrons, while 100 per cent was found in the filtrate at 188 millimicrons. The authors decided that in general it might be assumed that the smallest specific antibody of antipneumococcic rabbit serum corresponds to a pore size of 11 millimicrons, the smallest in horse serum to a size of 44 millimicrons, while both horse and rabbit antibodies have large specific aggregates corresponding roughly to a pore size of 88 millimicrons. Furthermore, the antibody of concentrated horse serum requires a pore size of approximately 176 millimicrons.

Goodner, Horsfall, and Bauer drew attention to the fact that the figures given are multiples of 11, which corresponds to the value of 11 to 12 millimicrons determined by Elford and Ferry[353] for the isolated form of normal horse pseudoglobulin.

Recent observations on the molecular size of pneumococcal antibody in immune rabbit serum, in immune horse serum, and in concentrated antibody solution are of importance not only in connection with differences in the characters of antibody as produced in animals of different species, but in emphasizing the range in surface area of the different types of antibody. Gram for gram, the pneumococcal antibody from the immune rabbit, because of its smaller molecular size and therefore its greater surface area, should exhibit greater combining power for homologous antigen than the antibody of the larger molecule of immune horse serum and that of the still larger aggregate present in concentrated antipneumococcic serum.

THE SEPARATION OF ANTIBODIES FROM IMMUNE SERUM

Inasmuch as the concentration and purification of pneumococcal antibodies will be described in detail in Chapter XV, only the principles involved in the separation of specific substances from immune serum will be dealt with in the present chapter. Gay and Chickering[508-9] took advantage of the particulation that takes place when cellular substances of Pneumococcus are brought into contact with homologous immune serum. From the antigen-antibody precipitates the antibody was then recovered by the use of suitable physical and chemical agents. The method was adopted, with modifications, by Huntoon and his associates[665-6, 668-9] and the use of specific precipitates has been developed as furnishing a source of pneumococcal antibody. Another basic method is founded on the relative insolubility of the immune proteins in water at their isoelectric point, as well as their insolubility in water in the absence of electrolytes. The use of protein coagulants such as ammonium and sodium sulfates, so valuable in the concentration and

refinement of antitoxins, and a similar use of alcohol, have re-
ceived wide application in the study of the chemical nature of im-
mune substances against Pneumococcus and especially in the quan-
tity concentration of antipneumococcic serum. The affinity of the
immune bodies for metallic salts also affords a method for the iso-
lation of antibody. Now ultracentrifugation and ultrafiltration
promise means of separating pneumococcal antibody from the ac-
companying components of immune serum.

THE ESTIMATION OF PROTECTIVE ANTIBODIES

In the quantitative estimation of the content of protective anti-
body in antipneumococcic serum several variables are encountered
in the culture, the serum, and the test animal employed. Inasmuch
as methods for measuring the therapeutic potency of antipneumo-
coccic serum will be discussed in the chapter dealing with serum
production, brief mention may be made at this point of some of the
factors which may influence the accuracy of the determination.
Enlows[366] pointed out the necessity of using cultures in the phase
of active growth or in the early stationary phase in order to avoid
lag on transfer. The hydrogen ion concentration of the medium in
which the pneumococci are grown should be such as to ensure the
presence of cocci at the height of vitality. The virulence of the
culture should be maintained at a high level. Felton[402] standard-
ized the infecting dose of culture by plate counts and employed a
dilution of culture representing 500,000 fatal doses of organisms.

Felton (1926)[399] discovered in antipneumococcic serums for
Types I, II, and III, a substance that was lethal for mice. Later,
Felton and Bailey[419] pointed out that from immune serum precipi-
table residues could be isolated that had an antagonistic action on
the neutralization of protective antibody by capsular polysaccha-
ride. Furthermore, the amount of soluble specific substance oper-
ated to cause a zonal effect in the estimation of protective action.

The mouse, on account of race, age, weight, and physical condi-
tion, presents an array of variable factors in the response to the

protective action of specific antipneumococcic serum. These factors, described by Felton[405] for the mouse, and by Goodner,[532-4] and Goodner and Miller [540] for the mouse and rabbit, have already been mentioned in the chapter on antigenicity and need not be repeated.

For elimination of the variables in the animal host, for simplicity and rapidity of performance, the newer methods based on specific precipitation present many advantages over the mouse protection test for the quantitative determination of protective antibody in antipneumococcic serum.

Other Immunological Phenomena

GROWTH OF PNEUMOCOCCI IN SPECIFIC IMMUNE SERUM

The growth of pneumococci in immune serum was observed by Metchnikoff[894] in 1891, and Denys (1897)[312] reported that pneumococci grow as well in immune as in normal rabbit serum. In the experiments of Rosenow (1904),[1159] pneumonic blood was found to possess no bactericidal properties for Pneumococcus, while viable organisms could be propagated from agglutinated masses of the cocci in the serum of pneumonia patients. An initial lag in the multiplication of pneumococci planted in the serum of artificially immunized animals, followed by active proliferation of the cells, was described in 1920 by Bull and Bartual.[177] Nicolle and Césari (1926)[1008] obtained better growth in Martin bouillon containing specific homologous serum than in the same medium to which heterologous immune or normal serum had been added. The ability of pneumococci to grow in homologous anti-S and anti-R immune serum and the effects of the serum on the growth of the cocci has been described in Chapter V.

ANTIBLASTIC IMMUNITY

An action of antipneumococcic serum apparently not definitely attributable to any of the known specific antibodies was described

in 1916 by Dochez and Avery,[320] who found that immune serum temporarily inhibited the multiplication of pneumococci and, at the same time, depressed the proteolytic and glycolytic functions of the bacterial cell. The retardation of growth and inhibition of metabolic activity of the cocci was ascribed by the authors to anti-enzymatic substances in antipneumococcic serum, and to the phenomenon they applied the term "antiblastic immunity." Blake (1917)[124] differed with Dochez and Avery in the explanation of the effect, and since it was found that the inhibitory action of serum paralleled the agglutinative power and, moreover, since serum exhausted of its agglutinins no longer interfered with the metabolic activities of the cell, Blake denied the participation of any anti-enzymatic principle in the phenomena. Barber (1919)[78] observed the inhibitory effect on the vital activities of pneumococci of whole fresh blood, coagulated plasma, and the serum of normal or immune horses and pigeons and, while attempting no analysis of the factors involved, believed that the action was antiblastic in nature. Bordet (1931),[140] noting the effect of normal rabbit serum on cultures of Pneumococcus, as manifested by changes in the morphology of the cocci, queried whether the disturbance in the metabolism of the organisms could be due to alexin or to the production of acid in cultures containing rabbit serum. The influences displayed by immune serum on the vital functions of pneumococci, as just described, are probably the same as those leading to the dissociation and degeneration of the pneumococcal cell.

IMMUNOLOGICAL RELATIONSHIPS BETWEEN PNEUMOCOCCUS AND OTHER MICROBIC SPECIES AND UNRELATED SUBSTANCES

Analogous to the apparent serological relations already described in Chapter VIII existing between Pneumococcus and such varied entities as gum arabic, *Bacillus coli*, *Leuconostoc mesenteroides*, Friedländer's bacilli, and tubercle bacilli, are the cross-reactions demonstrated by Sugg and Neill (1929)[1355] between yeast and Type II Pneumococcus. The reader may recall that

Mueller and Tomcsik (1924)[938] had described a complex carbohy-
drate prepared from yeast that bore certain chemical resemblances
to the capsular polysaccharide of Pneumococcus. It remained for
Sugg and Neill[1356] to demonstrate that the resemblance extended
to the immunological behavior of these two representatives of
Schizomycetes and *Saccharomycetes*. Pneumococci of Type II, but
not of Types I or III, were agglutinated by serum from rabbits
immunized by injections of a certain variety of yeast, and filtrates
of young, unautolyzed broth cultures of the same type of pneumo-
cocci invariably precipitated potent antiyeast serum. The anti-
yeast serum, furthermore, protected normal mice against Type II
pneumococci as well as the average specific antiserum produced in
rabbits for this type. The reciprocal reaction of Type II anti-
pneumococcic serum with yeast was not so definite, since serum from
many normal rabbits was capable of agglutinating yeast. In a 1 to
5 dilution, Type II antipneumococcic serum gave clean-cut agglu-
tination. The results of absorption experiments with both the anti-
yeast (rabbit) serum and the Type II antipneumococcic (horse)
serum were the same as those usually obtained in analogous experi-
ments with immunologically related, but not identical, species of
bacteria.

The protection against infection with Type II pneumococci con-
ferred upon mice by injection of suspensions of heated yeast cells
was practically the same as that obtained by vaccination with
Type II pneumococci themselves, with the exception that yeast
evoked immunity to Type II and not to Type I or Type III or-
ganisms. The mutual reactions of yeast and Type II pneumococci
were further demonstrated when Sugg and Neill (1931)[1356] em-
ployed semi-purified carbohydrate antigens from both yeast cell
and pneumococci mixed with homologous antiserums. Antiyeast
serum precipitated with, and sensitized guinea pigs to the Type II
antigen but was not active with antigens from strains of Type I
and III pneumococci. The Type II antigen was almost as reactive
against antiyeast serum as against homologous antiserum. Con-

versely, Type II but not Type I or Type III antipneumococcic serum reacted with yeast carbohydrate antigen but not to the degree observed in the case of antiyeast serum and pneumococcal carbohydrate. The action of different samples of antiyeast serum of equal potency as to reactivity with yeast antigen varied greatly when tested against pneumococcal antigen.

Summary

The substance of this chapter may be recapitulated as follows: The introduction into the bodies of animals of suitable species of pneumococci and some of their natural components augments or brings into being an array of specific immune substances that serve to protect the animal against the invading cocci and that can be demonstrated by appropriate serological and other immunological reactions. The immune substances thus evoked comprise agglutinins, precipitins, opsonins or tropins, and complement-fixing and protective antibodies.

Agglutinins, arising as a result of artificial immunization or appearing in the blood of pneumonia patients at or near the time of crisis, are easily demonstrated: the specific agglutination reaction takes place between the intact pneumococcal cells, whether living or dead, and the homologous antibody in the serum used. The reaction varies from strict type-specificity to a broader species-specificity, depending upon the nature of the antigen employed in the production of the immune serum and that of the hapten participating in the reaction. The somatic protein of the cocci and of degraded or R forms of the organisms engenders agglutinins reactive with all types of pneumococci, whereas the intact, virulent forms of pneumococci or their unimpaired capsular constituents evoke agglutinative substances strictly specific for the type of antigen or component supplying the antigenic stimulus. In the latter instance the reaction is one between the specific capsular polysaccharide of the organism and its corresponding specific, immune-serum globulin. Because of the simplicity of the technique required

for the detection or quantitative determination of agglutinins in immune serum, the agglutination reaction finds a wide use in the identification of members of the types within the species.

The precipitin reaction, involving as it does the interaction of hapten and immune body in solution instead of the reaction between formed microbic elements and specific serum and, moreover, being independent of the participation of the immunological factors possessed by a living, immune animal, is susceptible to the application of mathematically accurate quantitative measurements. By means of the precipitin reaction, it is possible to determine with great exactness the quantity of hapten in a given solution or the amount of immune nitrogen—that is, the amount of specific antibody in immune serum. Because of the fortunate, close parallelism existing between the content of precipitin and protective antibody in any particular serum, a quantitative estimation of the therapeutic value of antipneumococcic serum can be made in the test tube instead of in experimental animals.

The greater economy of means required in the reaction and its greater accuracy when properly performed have led to far more detailed studies of the phenomenon which, in turn, have supplied many clues to the mechanism operating in immunity to Pneumococcus. The chemist has thus been given the means of learning the very chemical radicals in the precipitinogen and the globulin fraction of immune serum which join to produce the precipitate and presumably which participate in the other immunological manifestations. The specificity of serological precipitation depends on the interaction of the capsular polysaccharide and immune globulin; and the basic process is the same as that in the phenomenon of agglutination.

The method of complement fixation offers no advantages over other serological methods for the demonstration or measurement of pneumococcal antigen and antibody. It has, however, revealed and enabled one to study the striking difference in the nature of specific antibodies in immune rabbit serum and immune horse serum.

Substances that neutralize the hemotoxin of Pneumococcus have been discovered in specially prepared immune serum, and some evidence has been presented that substances capable of inhibiting the action of the poisonous products of Pneumococcus may arise in the immunization of animals with the so-called pneumococcal toxins.

In a true sense there are no specific bactericidins for pneumococci. The destructive effect on pneumococci of body tissues is referable to a more complex play of biological elements.

There exist in the normal animal body, and to a much greater degree in the body of immune animals, substances that render pneumococci susceptible to the phagocyting action of mobile leucocytes and the fixed body cells. Disregarding the confusion of terms, the opsonins or tropins act on the bacteria and not on the phagocytic cells and it is by means of the intervention of the normal or immune opsonins that, under suitable conditions, pneumococci may be destroyed in the test tube or in the body of the infected animal.

The combined phenomena of agglutination, precipitation, and opsonization are the complement of forces mustered by the animal body for protection against invading pneumococci. The assumption is supported by the close correlation found to exist between the various antibodies concerned in antipneumococcal immunity. The specific protective action of immune serum can be strikingly demonstrated in laboratory animals and the type-specificity and the degree of this protective action can be measured by *in vitro* methods. Through chemical studies, the protective antibodies have been located in the globulin fraction of immune serum and by both chemical and physical manipulations they may be isolated in relatively pure form. Recent advances in these technical procedures have made possible actual measurements of the molecular size of pneumococcal antibodies and are leading to a clearer conception of their chemical composition.

There remains to be explained the significance of the immunological relation of pneumococcal antigens and antibodies to those

of alien microbic species, as well as their relation to apparently biologically unrelated substances in both animal and vegetable tissues. The solution of these problems will go far to explain dependence of immunological action on chemical composition.

CHAPTER XII

HOST RESPONSE TO ANTIGENIC ACTION
OF PNEUMOCOCCUS

Normal barriers to the invasion and multiplication of pneumo-cocci in the animal body; the immunological reaction naturally stimulated by pneumococcal infection and the reaction artificially aroused by the administration of pneumococci, their constituents, or derivatives; the somatic manifestations of the immune state; and the utilization by the body of antibodies passively acquired.

SOUND skin and healthy mucous membranes are impediments to the entrance of Pneumococcus into the animal body. Under-lying these tissues, as a line of secondary defense, are the leuco-cytes, which with normal auxiliary elements of the blood may in-gest and destroy the invading cocci. The localization or the sys-temic distribution of the organisms depends on the vigor of the phagocytic response and on the functional capacity of the body to elaborate specific antibodies in response to the antigenic stimu-lus provided by the invading microorganisms.

Total failure of the defenses means death but, with only a par-tial lack of antagonistic factors, the animal may pass through the rigors of pneumococcal disease and thereby acquire greater, if temporary, resistance to subsequent attacks. An analysis of the protective mechanism of the animal economy against pneumo-coccal infection will be attempted in the present chapter.

Natural Immunity

IN ANIMALS

A reason for the behavior of various normal animals toward pneumococcal infection was sought by Tchistovitch (1890),[1381] who subjected dogs, rabbits, and mice to subcutaneous, intra-tracheal, and intraocular inoculation with *Streptococcus lanceo-*

latus (Pneumococcus). The animals were killed during successive stages of infection and the lungs examined for cellular changes. The differences in the physiological response corresponded with the natural susceptibility of the animal species to infection. In non-refractory animals, the cocci caused only a feeble local inflammatory reaction with little phagocytosis, and the leucocytes neither engulfed the organisms nor inhibited their growth. In refractory animals, on the contrary, a more or less pronounced local inflammatory process developed, with leucocytic migration and accompanying phagocytosis. When the injections were made into the anterior chamber of the eye, there appeared to be no difference in the reaction of the aqueous humor of the animals of the two classes, since the fluid served as a medium for the growth of the injected cocci.

Opposed to the observations of Tchistovitch were those of Behring and Nissen,[98] published in the same year, who could find no differences in the bactericidal properties of the serum of mice, rats, and rabbits. The inability of the serum of the animals to affect pneumococci was in marked contrast to their destructive action on anthrax bacilli. Wadsworth[1455] also (1903) was unable to discover any parallelism between the action on pneumococci of serum from normal animals and the natural resistance of the animals to pneumococcal infection. When tested by agglutination and precipitation methods, the serums in low dilutions showed no significant differences.

Natural immunity of the pigeon to Pneumococcus, according to Strouse (1909),[1345] was due to high normal body temperature and not to any specific tissue reaction. Employing both *in vitro* and *in vivo* methods for studying phagocytosis, Strouse could detect no difference in the reaction of pigeons and of mice to experimental infection. Ungermann[1434] also studied the phenomenon of phagocytosis occurring after the addition of serum of various animal species to leucocytes of animals of homologous and heter-

ologous species. While in the case of the rabbit and mouse no phagocytosis was observed when virulent pneumococci were used in the test, the destruction of avirulent strains seemed to parallel the resistance toward the particular strain possessed by the animal whose serum was tested. While Dold (1911)[323] could demonstrate no substances antagonistic to Pneumococcus in the serum, plasma, or whole blood of normal mice and rabbits, by treating rabbit leucocytes by the method of Schneider, extracts were obtained which Dold claimed had definite killing power for pneumococci.

Robertson and Sia (1923)[1144] devised an accurate method for demonstrating growth-inhibitory and bactericidal action on Pneumococcus of normal serum-leucocyte mixtures. The ingredients of the mixture were added in known quantities and mechanically agitated by rotation and oscillation. A combination of serum and leucocytes from resistant animals (cats and dogs) exerted not only a growth-inhibiting but also a bactericidal action on pneumococci. The serum-leucocyte mixtures of susceptible animals (rabbits and guinea pigs) showed no inhibitory effect. In subsequent communications, Robertson and Sia[1145-7] substantiated their earlier results. For example, the growth of pneumococci possessing low virulence for the cat was found to be markedly inhibited in mixtures of cat serum and cat leucocytes, since ten thousand times the number of pneumococci ordinarily sufficient to kill a mouse failed to infect after being exposed for twenty-four hours in the cat serum-leucocyte mixtures. Furthermore, virulent strains sensitized by contact with serum of animals resistant to Pneumococcus were actively phagocyted, not only by the homologous leucocytes, but also by the leucocytes of other resistant animals and of susceptible animals. However, pneumococci exposed to the action of serum from susceptible animals were not taken up by leucocytes of either the resistant or susceptible animals. The serum of all the resistant animals tested—dog, cat, sheep, pig, and horse

—showed marked opsonic properties which were absent from the serum of animals of such susceptible species as the rabbit, guinea pig, and man. In contrast to the activity of serum there appeared to be no essential difference in the phagocytic power of the leucocytes from the various animals. The influence of the age of the animals on the pneumococcidal properties of the blood was shown by the fact that mixtures of adult rabbit serum with either adult or young hare leucocytes exerted pronounced growth-inhibitory and coccidal action, whereas mixtures of serum from immature hares with leucocytes from either adult or young rabbits completely lacked any similar action. In the natural defense mechanism against pneumococcal infection, it appears that the serum contains the potential elements and that in a susceptible animal like the rabbit the elements develop with the growth of the animal.

The results reported by Robertson and Sia were practically duplicated by those of Woo,[1541] who found that rabbit serum-leucocyte mixtures possessed the power to kill avirulent pneumococci in relatively large numbers, but failed to inhibit growth of even minute quantities of virulent organisms, an observation also reported by Wright (1927).[1547] Woo found further that the serum of very young animals when mixed with leucocytes was powerless to affect cultures which were without virulence for mature rabbits. Bull and Tao (1927)[182] introduced citration for determining the antipneumococcic properties of whole blood. One per cent by volume of saturated, neutral sodium citrate delayed coagulation of the blood for twenty-four hours and did not inhibit the growth of pneumococci. The killing action of citrated blood was more potent than that of serum-leucocyte mixtures. When the method was applied to the blood of normal rabbits and chickens, Bull noted that it required one million times as many pneumococci to infect a given quantity of chicken blood as it did to infect the same volume of rabbit blood.

The hypothesis that it was the natural, humoral antibodies

which were responsible for the pneumococcidal action of serum-leucocyte mixtures received confirmation in a separate study by Sia (1927),[1268] in which it appeared that the active principle operative in the destruction of pneumococci in appropriate serum-leucocyte mixtures could be specifically absorbed from the serum, and that the native principle—the opsonin—was type-specific in its selective action.

It was found by Kelley (1932),[701] as Robertson and Sia had previously discovered, that normal swine serum possessed the property of protecting mice against infection with virulent pneumococci and of agglutinating both virulent smooth and avirulent rough strains of the organism. The protective action of swine serum, although slight, was evidently specific for type, since the protective antibody for one type of Pneumococcus could be absorbed by cultures of the homologous type without affecting the content of antibody for other types. Unlike the protective substances in specific immune serum, those in normal pig serum were thermolabile and disappeared after a few weeks' storage in the cold and, moreover, they could be removed from the serum by absorption with avirulent rough pneumococci. Another difference between normal swine serum and specific immune serum lay in the fact that although the former was type-specific in mouse-protective action, when mixed with soluble specific substance no precipitation took place, nor was the protective action inhibited by the carbohydrate. Kelley also confirmed Robertson and Sia's observation that swine serum shows type-specific agglutination for pneumococci, and that agglutinins for one type may be specifically absorbed, leaving agglutinins for other types undisturbed. Inasmuch as normal pig serum agglutinates avirulent, rough forms of pneumococci and the agglutinative property is not destroyed by heating at 56°, Kelley assumed that this property probably depends on factors other than those responsible for agglutination of smooth forms of Pneumococcus and for protective action in mice.

In 1929, Sia[1269] presented further evidence in support of the view that humoral defensive elements play an important part in natural immunity to Pneumococcus. Mice were given intraperitoneal injections of serum from the pig and four hours later actively growing cultures of virulent Type I pneumococci were injected into the animals. Control mice were similarly injected with serum from susceptible animals (the rabbit and guinea pig) and then inoculated with the same culture. Normal swine serum protected the mice against ten thousand minimal lethal doses of culture, whereas serum from the susceptible animals afforded no protection to the test animals. Pig serum also protected mice against virulent Type II pneumococci and to a lesser degree against Type III organisms. Moreover, absorption experiments again demonstrated that the protective substance was type-specific.

Le Guyon (1931)[796] investigated the causes of the different reactions exhibited by rabbits and guinea pigs to pneumococcal infection. The technique employed consisted in inoculating the animals intraperitoneally with a broth culture of virulent pneumococci and in examining peritoneal fluid withdrawn at various intervals after injection. Four hours after inoculation, mice developed a marked cellular reaction. Along with many cocci there were always a large number of macrophages of the clasmatocyte type. There was intense phagocytosis by the polymorphonuclear leucocytes and macrophages, but in spite of the reaction the animals developed septicemia and died within eighteen to twenty hours. In guinea pigs four hours after inoculation, the reaction was feeble. Some polymorphonuclear cells and some free pneumococci were present in the exudate, but there was only occasional evidence of phagocytosis. Sixteen hours later, the number of organisms and polymorphonuclear leucocytes had greatly increased, but lymphocytes and macrophages were rarely seen. The pneumococci, some of which were phagocyted, were clustered about the polymorphonuclear cells and appeared to be in a degenerated condition. Le Guyon therefore concluded that the initial refrac-

tory state in the guinea pig was due to the bactericidal property of the serum.

Bordet (1933)[141] believed that in addition to the leucocyte-serum complex there were other humoral factors which accounted for the unequal susceptibility of animals of different species. The action of the hypothetical factors was manifested by the altered appearance of pneumococci grown in the serum of different animals. The principles appeared to be thermolabile, could be absorbed by aluminum hydroxide, and accompanied the euglobulin when serum was separated into its protein fractions.

A type of resistance, quite independent of strictly immunological processes, is that possessed by the rabbit against the majority of strains of Type III Pneumococcus. An experimental infection can be induced in the rabbit by intradermal injection of many strains of this serological type but the infective process is aborted and the animal recovers.

In the early stages of the infection the leucocytes are powerless to engulf the cocci but, as the body temperature of the animal rises to 104° or higher as a result of the infection, the organisms lose their capsules and are avidly phagocyted. One of the determining factors in the inability of many Type III pneumococci to produce fatal infection in the rabbit is the susceptibility of the organisms to the high temperature generated in the rabbit. A detailed discussion of the experimental evidence on which the above statement is based is to be found on pages 207 to 210 in Chapter VI.

IN MAN

The presence of normal precipitin for Pneumococcus in the blood serum of man was reported by Wadsworth (1903)[1455] but its content was low, since in serum in a dilution of 1 to 10 or more the action of the native precipitin was not evident. Rosenow (1904)[1159] denied that fresh normal blood or serum had any bactericidal influence on Pneumococcus. Neufeld and Haendel (1910)[990] demon-

strated the protective action of normal human serum for the mouse, while Much* reported that normal human serum, and to a still higher degree, plasma, contained thermolabile bactericidal substances for Pneumococcus.

Dold (1911),[323] testing by means of plate cultures the action of serum, plasma, and whole blood of normal human beings and of patients ill with diseases other than pneumonia, claimed to have demonstrated definite pneumococcidal effect. The action, stronger in blood than in plasma or in serum, varied from slight inhibition of bacterial growth to actual killing of the organisms.

According to Clough (1924),[243] normal human beings may possess in their serum substances capable of protecting mice against infection with pneumococci of Types I, II, and III. The degree of protection, when demonstrable, was usually slight, but in five instances was sufficient to save the animal from subsequent inoculation with 1,000 to 100,000 minimal fatal doses of pneumococci. Protective action against one type of organism was not necessarily accompanied by similar action against representatives of other types, nor was the property associated with the presence of any agglutinins or opsonins, since the latter antibodies were absent from the serums tested.

Burhans and Gerstenberger (1924)[190] tested the serum of infants and maternal parents for protective power against Type I, II, and III strains of Pneumococcus. The serum of approximately 40 per cent of the parturient mothers protected mice against inoculation with organisms of the three fixed types, while samples of serum from only about 30 per cent of the infants exhibited similar properties. The authors decided that the low incidence of lobar pneumonia during infancy was probably not due to an immunity to the fixed types of pneumococci.

Ash and Solis-Cohen (1929)[26] believed that differences in susceptibility to pneumonia among human beings could be determined by the growth-inhibitory and pneumococcidal action of

* Quoted by Neufeld and Schnitzer.

whole blood and, furthermore, that the reaction was dependent upon the presence of leucocytes.

Analogous differences in resistance were observed by Robertson and Cornwell (1930).[1143] In a study of the pneumococcidal action of normal human serum-leucocyte mixtures for freshly isolated strains of pathogenic pneumococci, the authors ascertained that human beings as a group possess in their blood well-marked destructive properties for all types of Pneumococcus studied. Individuals, however, exhibit wide variations in reaction against different types, ranging from strong killing effect for organisms of one type to no action or slight effect against strains of another type. In the light of the results of previous experiments in which actual resistance of animals to pneumococcal infection was determined, Robertson and Cornwell interpreted the findings as meaning that human beings in general possess a considerable degree of natural immunity to all types of Pneumococcus, but that some individuals may be susceptible to one or more types and at the same time be resistant to other types.

By a method employing whole blood, Ward[1480] found that the phagocytic titer against the first three types of pneumococci varied through a wide range in different normal human subjects. Similar results were obtained in the same year by Sutliff and Rhoades,[1363-4] who measured the pneumococcidal power of normal human blood by a modification of the method of Robertson and his co-workers. Whole blood, to which heparin was added in small amounts as an anticoagulant, was mixed with pneumococci and rotated in an apparatus devised by the authors. Parallel determinations of the protective power of the samples were carried out. The blood of seventeen out of twenty-seven hospital patients who had not had lobar pneumonia killed from one hundred to ten thousand virulent Type I pneumococci. When mouse-protective power and pneumococcidal power were compared, it was found that six subjects possessed both properties and that the serum of ten individuals was bactericidal but not protective, while the serum of

three subjects exhibited neither property. In another communication, Sutliff with Finland[1360] reported that the incidence of pneumococcidal power and of other type-specific antibodies varied with the age of the subject as well as with the different types of Pneumococcus. The killing action of normal human serum was most frequently observed with organisms of Type II, was rarest for Type I, and was intermediate for Type III strains.

Gundel,[567] in 1932, investigated the presence of pneumococcidins, protective antibodies, and agglutinins in the blood of normal adults and of healthy nurslings and children. The blood of adults frequently showed the presence of definite amounts of antibacterial antibodies, whereas no protective or agglutinative action was observed with the serum of new-born babies and young children. Gundel concluded that antibody formation began in normal children toward the end of the second year of life. Variations in the titer of humoral immunity were manifested in a third or a fourth of the subjects, there being an increase in the case of some individuals and a decrease in others. In the majority of instances the action was specific, since the altered reactivity toward organisms of one type was not accompanied by a similar change toward other types of pneumococci.

An experimental analysis of the factors responsible for the pneumococcidal action of human serum convinced Ward and Enders[1484] that in normal human serum virulent pneumococci may be prepared for phagocytosis by two separate antibodies acting in conjunction with complement. One of the substances is probably the type-specific anticarbohydrate antibody reacting with the capsular polysaccharide of Pneumococcus; the other is probably also a type-specific antibody, but quite distinct from the former and, therefore, reacting with a different antigenic constituent of the bacterium. In normal human serum heated to 56° the two antibodies may, after prolonged contact with the organism, promote phagocytosis of pneumococci without the adjuvant action of complement. While the two antibodies are equally effective in the

phagocytosis of twenty-four-hour cultures by normal blood, the anticarbohydrate antibody tends to predominate as the pneumo-cocci approach the state in which they exist in the animal body. Enders and Wu[362] later reported (1934) that the opsonic titer of normal human serum could be practically eliminated by the addition of the A carbohydrate, that is, the acetylated capsular polysaccharide of Pneumococcus.

Naturally Induced Immunity

SPECIFIC ANTIBODIES IN THE BLOOD DURING PNEUMONIA

Pneumococcus, upon entering the animal body and inciting morbid processes in the organs and tissues, by virtue of its several components arouses or stimulates physiological functions latent or active leading to immunity, and the products of the freshly activated functions may be detected and measured by the various serological reactions. The first intimation that immune substances arise in man as a result of pneumococcal infection came from the work of the Klemperers,[725] who discovered that serum taken from pneumonia patients after crisis displayed curative properties for experimentally infected rabbits. Similar protective antibodies were observed in the serum of pneumonia patients by Römer,[1155] in some cases several days after the onset of the disease. Then Neufeld and Haendel[990] succeeded repeatedly in demonstrating not only that there was a decided increase in protective substances for the mouse in the serum of pneumonia convalescents soon after crisis, but that the action was specific for heterologous as well as for homologous strains of pneumococci. The authors, therefore, were convinced that the crisis in lobar pneumonia depended upon the formation and specific action of antibodies. Seligman (1911),[1253] on the contrary, found no difference in the degree of protective action of serum taken before or after the critical period. Strouse (1911),[1346] using the Neufeld technique, was unable to detect opsonins in heated post-critical serum. However, after sensitizing the pneumo-

cocci with the serum, as recommended by Lamar, Strouse experi-
enced no difficulty in obtaining phagocytosis.

Eggers (1912),[349] by the plate method of determining bacteri-
cidal action, found increased antipneumococcal properties devel-
oping in the serum of pneumonia patients at or shortly after crisis
and lasting for variable periods thereafter. Cases in which the
apparently characteristic increase of bactericidal power did not
occur presented irregularities either in course or in termination.
Dochez (1912)[315] was successful in proving the presence of sub-
stances protective for mice in the serum of patients ill with lobar
pneumonia. All but one of the serums tested showed the ability to
protect, the reactive substance appearing in only a few instances
before the time of crisis, while in some other cases the protective
power appeared to persist as long as the patient was under obser-
vation. In some patients, protective antibodies either became evi-
dent at some time after crisis or could not be demonstrated at any
period of the disease. Dochez concluded that the appearance of
specific protective substances in the serum of patients ill with
lobar pneumonia suggested that these bodies might play a part in
the mechanism of recovery.

In the next year, Clough[240] reported the demonstration of
protective antibodies, specific for the infecting strain of Pneumo-
coccus, in the serum of the majority of patients after crisis or ly-
sis. Phagocytic activity of the serum ran closely parallel with pro-
tective power for mice. In addition to protective antibodies and
opsonins, Lacy and Hartmann (1918)[770] reported that specific
agglutinins usually appeared in the serum of pneumonia patients
during or shortly after defervescence. In a second report, Clough
(1919)[242] stated that the serum of 85 per cent of the patients ill
with acute lobar pneumonia whom he had studied showed positive
phagocytic activity after crisis or lysis, and that the serum of 79
per cent of the cases showed agglutinative activity. In a few in-
stances, positive results were obtained twenty-four hours or less

before crisis. While phagocytic and agglutinative activity were observed for all the pneumococcal types tested, the reaction was always strictly limited to organisms homologous with those with which the patient was infected.

Müller (1923)[939] was inclined to ascribe to humoral antibodies only a minor part in recovery from pneumonia. His belief was based on negative results in tests for bactericidal power of serum from the majority of patients studied, and also on the fact that the author failed to observe any increase in the property during the course of the disease. Adler (1923),[3] on the contrary, reported that the serum of pneumonia patients developed the highest content of bacteriotropic substances at the time of crisis. However, Baldwin and Rhoades (1925)[68] contended that recovery in pneumonia is associated with the appearance of specific antibodies in the blood, and that the antagonistic action of the protective substance is revealed by the fact that pneumococci and protective antibody rarely appear simultaneously in the circulating blood. The presence of protective substance in the blood practically always precluded a concurrent bacteriemia, but it did not in every case prevent toxemia, relapse, or the development of complications. Nevertheless, protective activity of the patient's serum appeared to be an important factor in overcoming pneumococcal infection.

With refined technique for determining the antagonistic action of serum from pneumonia patients, Sia, Robertson, Woo, and Cheer (1925)[1274] found that in all cases of pneumonia studied the serum at or soon after crisis possessed the power to inhibit growth of pneumococci in rabbit serum-leucocyte mixtures. Before crisis, serum either lacked the property or exhibited it only to a slight degree. The titer reached its highest point three or four days following crisis and then gradually diminished, although in one patient recovering spontaneously from pneumonia due to Type I Pneumococcus antipneumococcic substances were still demonstrable in the blood for seventy days. In cases terminating fatally no

such substances could be found at any time during the course of the disease. In a second paper, Sia with Robertson and Woo[1273] reported that the same conditions prevailed in pneumonia caused by pneumococci of Types I and II and Group IV. At the critical period, opsonins and agglutinins were also demonstrable in the blood.

Other evidence of the production of an altered condition related to the immune state developing in the body of pneumonia patients, was the abolition of reactivity of the skin to pneumococcal filtrates and extracts, as described by Herrold and Traut (1927).[637] The serum of patients failing to react to the skin test partly or completely neutralized *in vitro* the antigenic substance in the active extracts and conferred protection upon mice.

The time factor in the appearance of protective substances and agglutinins in the course of lobar pneumonia was investigated by Lord and Nesche (1929).[828] The authors were unable to find these antibodies in the blood of patients before a fall in temperature by crisis or lysis, but could demonstrate their appearance and continued presence as soon as defervescence took place. Lord and Nesche attributed importance to the action of protective antibodies in bringing about recovery, since a large proportion of pneumonia patients with these substances in the blood conquered the disease, while the majority of patients without protective antibodies in their serum died. However, that immune bodies may be present in the early stages of untreated pneumonia is indicated by the experiments of Ward,[1480] who observed that the phagocytic titer of whole human blood was comparatively high against the infecting organism. For Ward, the fact pointed to a local rather than a general lowering of resistance in infection with Pneumococcus.

In 1931, Lord and Persons,[838] continuing the study of the production of specific antibodies during pneumococcal pneumonia, reported that although in general the appearance of protective substance coincided sharply with the fall in temperature of the pa-

tient, the antibody might appear spontaneously in the blood serum as early as the third or fourth day of the disease and crisis and recovery might be delayed until the sixth to the tenth day. However, recovery might occur without demonstrable protective substance in the blood of patients whose serum later developed protective properties. According to Lord and Persons, the amount of antibody appearing in the course of pneumococcal pneumonia was small and it might be present in the blood concurrently with septicemia. The formation of protective substances by the patient gave no assurance that the infection would not progress to a fatal termination.

In empyema fluids of pneumococcal origin, Floyd (1920)[456] demonstrated the presence of specific precipitin and of much smaller amounts of agglutinin, and considered that their occurrence was generally a favorable prognostic sign. Later, Finland[441] reported (1932) that sterile pleuritic exudates from patients with lobar pneumonia contained actively acquired antibodies similar to those developing in the blood serum.

A property of the serum of pneumonia patients which had escaped previous notice was the capacity to precipitate in high titer the non-protein somatic substance—the C Fraction—derived from pneumococci. Tillett and Francis (1930)[1409] tested serum obtained from patients during illness and convalescence for antibodies specifically reactive with this chemically distinct carbohydrate of Pneumococcus. The results, when correlated with the course of the disease, demonstrated differences in the occurrence of each qualitatively distinct antibody. Strangely enough, the precipitating action of the serum on the somatic carbohydrate developed in the very early stages of the disease, only to disappear at the time of crisis. Precipitation of Fraction C was not limited to the serum of individuals ill with pneumococcal infection and, in the few cases available for comparative tests, definite reactions were obtained only in streptococcal and staphylococcal infections and in acute rheumatic fever.

IMMUNE SUBSTANCES IN SPUTUM

Lord and Nye (1921)[831-3] observed that purulent sputum col-
lected during life and the pulmonary exudate obtained at necropsy
from the later stages of lobar pneumonia commonly eroded the
surface of Loeffler's blood serum and, in a separate communication,
Nye[1021] reported that washed cellular suspensions of pneumonic
lungs, previously preserved with chloroform and toluene, contained
a proteolytic ferment, derived chiefly from the leucocytes of the
exudate. Eddy (1928)[348] found that filtrates from sputum ob-
tained after crisis from patients with lobar pneumonia conferred a
certain degree of protection on mice, with sometimes only a delay
in the time of death. The effect was never produced by filtrates of
sputum obtained before crisis or from fatal cases, nor was the fil-
trate active with organisms other than those of the type infecting
the patient. The sputum of two patients displayed proteolytic ac-
tion, but Eddy was unable to demonstrate bacteriophage in any of
the specimens of sputum. The observation recalls that of Dick
(1912),[314] who by noting the optical rotation of mixtures of serum
from pneumonia patients taken at the time of crisis found that a
decrease in optical rotation occurred at that time and not before
or after the critical period. Dick ascribed the phenomenon to pro-
teolytic activity of the serum.

ANTAGONISTIC SUBSTANCES IN PNEUMOCOCCAL EXUDATES

Proceeding from the conception that increased acidity in pneu-
mococcal cultures might find an analogy in the pneumonic lung,
Lord (1919),[826] upon testing the hydrogen ion concentration of
morbid exudates, found higher acidity in three of four cases than
that in the press juice of the unaffected lung. Lord suggested that
increase in acidity in the diseased pulmonary tissue might favor
enzymatic action as well as inhibit pneumococcal growth.

In further studies on pneumonic exudates, Lord with Nye
(1921),[830-1] demonstrated the presence of a proteolytic enzyme.
The enzyme remained active after eighteen months' preservation

and resisted heating at 65° for one hour, but was destroyed after heating for the same period at 75°. No dialysis of the enzyme could be demonstrated. The activity of the enzyme persisted in concentrations of sodium chloride varying from normal to thirty-two times normal. In exudates, antigenic substances were also found, evidently arising from the dissolution of pneumococci in the infected lung. The presence of specific precipitinogen was disclosed when the exudate was mixed with homologous antipneumococcic serum. After testing extracts of affected lungs, Lord and Nye[833] further concluded that the pneumonic lung contained a soluble substance inhibiting agglutination of fixed types of pneumococci by homologous serum. An analogous substance was found by Ward (1932)[1483] in the filtrate of a lung obtained at necropsy from a patient dying from pneumonia due to Type III Pneumococcus. The substance was similar to that present in somewhat lower concentration in broth cultures of Type III Pneumococcus which displayed powerful antibactericidal action.

Artificially Induced Immunity

The antigenic properties of Pneumococcus and the manifestations of the immunological reaction of the animal body to antigenic stimuli have already been treated in the text in some detail. However, in order that the presentation of the features of the animal hosts' response may be orderly and complete, a brief synopsis is made at this point of the factors that are chiefly concerned in the artificial production of the immune state.

ACTIVE IMMUNITY

The ability of pneumococcal materials, purposely introduced, to raise the resistance of susceptible animals to infection with Pneumococcus, with the accompanying elaboration of demonstrable specific immune substances or antibodies, is dependent upon the nature of the material employed and also upon the special racial and individual peculiarities of the animal treated. The more

closely the state of the antigen approaches that of the vigorous, living, virulent cell, the greater the specificity and the completeness, within certain limits, of the immunity and of the antibodies evoked. The robust organism with its protein and carbohydrate constituents in fully developed and unaltered condition exerts the greatest antigenic action. The cell should be living, or devitalized at the peak of its anabolic activities by heat or by such chemical agents as rob the bacterium of the ability to propagate without disturbing its chemical integrity. The greater the mass, within certain limiting zones, the more energetic the specific antigenic stimulation; while the proper spacing of injections and the route by which the antigen enters the body may affect the specificity and the quantity of antibodies produced.

The protein of the pneumococcal cell is antigenic only in the sense that its administration by parenteral routes results in the appearance of humoral antibodies specific for the bacterial species and not for the serological type of the organism injected. The capsular polysaccharide, besides orienting the antigenic action of its conjugated protein when its molecular configuration is undisturbed, is antigenic in itself, and it is this fraction of the pneumococcal cell that determines the type-specificity of the immune response. The somatic carbohydrate—the C Fraction—if it has any antigenic action, apparently has a subordinate and as yet unknown share in the immunizing action of Pneumococcus.

In an animal of a susceptible species, unaffected by any debilitating condition, the parenteral introduction and, to a much less degree, the oral administration of properly chosen pneumococcal antigens, decreases susceptibility to pneumococcal infection and arouses physiological functions latent in the cells of the body. These functions result in the extrusion into the circulation of substances corresponding in type to the kind of antigen administered, and demonstrable and measurable by the various serological reactions. In addition to the appearance of humoral antibodies, the somatic cells undergo changes in reactivity to pneumococcal ma-

terials. Of the immune substances engendered, the agglutinins ag-
glomerate the cocci and facilitate their removal from the circu-
lating blood and the precipitins combine with the polysaccharide
present in the capsule or released in the disintegration of the bac-
terial cell, while the opsonins render the organisms susceptible to
the phagocytic action of the leucocytes and of certain of the fixed
cells of the body.

The immunity thus established by artificial means may become
apparent within the space of a few days after the administration
of antigen and may persist for longer or shorter periods depend-
ing upon the total amount of antigenic material injected, the
spacing and repetition of the injections, and the ability of the
tissues to continue their special functions. Immunity to Pneumo-
coccus is, at best, a transient condition, and unless the tissues are
fortified by continued specific stimulation, the antibodies thus ar-
tificially induced shortly disappear from the blood and the animal
again becomes vulnerable to the pathogenic action of Pneumo-
coccus.

PASSIVE IMMUNITY

The blood or serum of an immune animal, whether transferred
through the placental circulation or artificially injected into the
body of a susceptible animal, carries its complement of antibodies
that serve to convert susceptibility into resistance; the degree of
resistance depends upon the potency of the serum in immune sub-
stances, the volume of serum, the route and frequency of adminis-
tration, and the capacity of the recipient to utilize the antibodies
so conferred. In the passively immunized animal, the type of im-
munity acquired corresponds in specificity to the immunity of the
donor.

References to the inheritance of immunity to pneumococcal in-
fection are singularly rare. By inference, it might be assumed
from the work of Irwin and Hughes[670] that native resistance may
be increased by selective breeding, but in that case the ability of

the animals to withstand infection is due to transmitted constitu-
tional factors unrelated to those which account for the specific
immune state. The report of Eguchi (1925)[351] is one of the few
communications dealing with this phase of immunity. The young
of female mice which, during the period of gestation and lacta-
tion, had received repeated intravenous injections of killed Type
I pneumococci were found to be immune to organisms of the same
type as those employed for immunizing the mother. The ability of
the mother to protect the offspring disappeared between the six-
teenth and twenty-eighth day after the last immunizing injection.
Eguchi believed that protection was conferred through the milk of
the immune mother. The results of attempts to transmit specific
immunity to the progeny by similarly treating the male parent
were inconclusive.

Another natural medium for the transference of specific immune
bodies is the serum of patients convalescent from pneumococcal in-
fection. Gundel (1931)[565] studied the curative action of conva-
lescent serum and while it was found to be inferior to the usual
therapeutic serums of animal origin, yet, according to Gundel,
specific therapy with human serum possesses advantages over the
use of animal serum in the avoidance of anaphylaxis and serum
disease. Other phases of passive immunity will be described in those
sections of the text where a discussion of the mechanism is espe-
cially pertinent to the particular subject under consideration.

Allergy and Anaphylaxis

In addition to rendering a susceptible animal immune to infec-
tion by Pneumococcus, the intact pneumococcal cell and its sepa-
rate or conjugated components possess the capacity to alter the
reactivity of the body tissues to derivatives of the coccus when
parenterally introduced. Animals so sensitized may develop acute
fatal anaphylactic shock when appropriately injected with cer-
tain of the derivatives of Pneumococcus, while the uterus and the

dermal tissues of animals thus treated display a newly established function when brought into contact with these products. Actively acquired hypersensitivity may be duplicated by the injection of normal animals with some forms of antipneumococcic serum.

The interaction between specific immune serum and the pneumococcal cell resulting in the formation of a substance capable of eliciting acute anaphylactic shock in the guinea pig was first demonstrated by Neufeld and Dold (1911).[981] Cocci sensitized with homologous antiserum and injected into guinea pigs regularly caused the acute death of the animals. The effect was ascribed by Neufeld and Dold to the production of anaphylatoxin.

ACTIVELY INDUCED SENSITIZATION

In 1911, Rosenow[1165] claimed that, by the subcutaneous, intravenous, intrapleural, and intraperitoneal injection of killed pneumococci or of filtered pneumococcal extracts into guinea pigs, he could so sensitize the animals that they responded with severe intoxication when the same bacterial extract was injected eight to twelve days later into a vein or into the heart. In a subsequent study, Rosenow (1912)[1166] was able to obtain highly toxic substances from Pneumococcus through autolysis, from Pneumococcus-leucocyte mixtures, and by the action of normal and of immune serum on the organisms. These toxic substances evoked in normal guinea pigs symptoms indistinguishable from the symptoms of immediate anaphylaxis. The action of the poisonous substances as well as of the preparations of Cole, Weiss, and others has been discussed in Chapter III, and undoubtedly is to be attributed to histamine-like protein-degradation products.

Clough (1915)[241] employed saline extracts of washed, dried, and ground pneumococci as sensitizing antigens and as the intoxicating agent. The extracts so prepared were sufficiently toxic to cause anaphylactoid reactions in normal guinea pigs. However, by precipitating the extracts with alcohol, Clough isolated a substance,

assumed to be protein, that was antigenic in the sense that it could sensitize normal guinea pigs and specifically intoxicate animals so treated.

The allergic state produced in guinea pigs by the intraperitoneal injection of killed broth cultures of Type I pneumococci was observed by Mackenzie (1925).[844] During the course of artificially induced, active immunity, the animals at times showed anaphylactic symptoms after the injection of the immunizing antigens. Bull and McKee (1929),[180] in an investigation of sensitization of the rabbit resulting from acute experimental infection with Type I Pneumococcus, found that the animals had acquired both dermal and systemic hypersensitiveness to pneumococcal autolysate. Sensitivity appeared within forty-eight hours after infection and persisted for at least four months, apparently reaching its height shortly after recovery from infection. Rabbits immunized by the injection of killed and living cultures also became hypersensitive to autolysates but not to so high a degree as did the animals recovering from infection.

Although Avery and Tillett (1929)[60] were unable to sensitize guinea pigs with the type-specific carbohydrates of pneumococci of Types I, II, and III, Tillett, Avery, and Goebel,[1407] in the same year, demonstrated the essential function of the carbohydrate fraction in determining the specific sensitizing and anaphylactic action of the carbohydrate-protein complex. Artificially prepared gluco-globulin and galacto-globulin possessed the property of actively sensitizing guinea pigs so that the guinea pigs, when injected twenty-one days later with sugar-proteins containing carbohydrate identical with that present in the sensitizing antigen regardless of the kind of protein with which it was combined, were subject to acute anaphylactic shock. Moreover, the unconjugated glucosides, although themselves incapable of inducing shock, inhibited the anaphylactic reaction when injected immediately prior to the introduction of the toxigenic sugar-protein into the specifically sensitized guinea pig. The protective, or anti-anaphylac-

tic action of the glucoside disappeared within two hours after in-
jection and, in order to elicit the phenomenon, the carbohydrate
had to be the same as that combined in the sugar-protein complex.

PASSIVE SENSITIZATION

From the collective experience of immunologists it becomes evi-
dent that some of the isolated constituents of the pneumococcal
cell are incapable of so sensitizing the guinea pig as to render the
animal susceptible to anaphylactic intoxication upon the paren-
teral introduction of Pneumococcus or its derivatives. However,
that an animal may be rendered highly sensitive to pneumococcal
substances by the injection of specific antipneumococcic serum is
supported by ample evidence. Weil and Torrey[1508] passively sensi-
tized guinea pigs by the injection of serum from pneumonia pa-
tients. When autolysates of pneumococci were added to the uterus
of the treated animal, positive contractions occurred only when
the serum employed originated from a case of pneumococcal pneu-
monia. By means of the same method, Zinsser and Mallory[1582] ob-
tained positive reactions with the uteri of guinea pigs passively
sensitized with antipneumococcic serum. More conclusive were the
observations of Tomcsik (1927),[1415] who demonstrated the ability
of the soluble specific substance of Pneumococcus to produce ana-
phylactic shock in passively sensitized guinea pigs. The purified
capsular polysaccharide of Pneumococcus, though apparently de-
void of sensitizing action, can induce rapid and fatal anaphylactic
shock when injected intravenously into guinea pigs passively sensi-
tized with the precipitating serum of rabbits actively immunized
with pneumococci of the homologous type, the reactions induced
being type-specific. Avery and Tillett,[60] who reported the observa-
tion, also drew attention to the fact that there was a complete ab-
sence of anaphylactic response to pneumococcal carbohydrate in
guinea pigs similarly treated with antipneumococcic horse serum.

As in their experiments on the production of active specific
sensitization with artificially prepared sugar-proteins, Tillett,

Avery, and Goebel[1407] demonstrated further the dependence of the
specificity of the anaphylactic phenomenon upon the carbohydrate
portion of these compounds. Guinea pigs passively sensitized with
the serum of rabbits immunized with an artificial gluco-globulin
exhibited typical anaphylactic shock when subsequently injected
with gluco-albumin; the serum of rabbits immunized with another
sugar-protein, galacto-globulin, similarly sensitized guinea pigs
to galacto-albumin. The reactions, in each instance, were specific
and depended for their specificity upon the carbohydrate com-
ponent and not on the protein fraction of the synthesized sugar-
protein. The authors found that anaphylactic shock could be in-
duced by uncombined globulin in guinea pigs passively sensitized
with either antigluco-globulin serum or antigalacto-globulin se-
rum, and that the globulin was similarly effective in animals ac-
tively sensitized with gluco-globulin or galacto-globulin. However,
the reactions provoked by globulin alone were dependent upon the
common protein present in the antigens and exhibited only species
specificity.

The haptenic participation of the A carbohydrate in the ana-
phylactic reaction was demonstrated by Enders (1930).[358] Guinea
pigs injected intraperitoneally with one to two cubic centimeters
of the anti-A rabbit serums used in the precipitin tests failed to
develop symptoms of anaphylaxis upon intravenous injection of
varying quantities of the purified specific carbohydrate. Nor was
the antibody against the nucleoprotein or rough autolysate pres-
ent in sufficient concentration in these serums to confer on guinea
pigs anaphylactic sensitivity to the substances. Again, no ana-
phylaxis developed in animals treated with the serum when autoly-
sates derived from virulent strains of either Type II or Type III
pneumococci were introduced intravenously. Type I anti-A serum,
however, regularly conferred upon guinea pigs a very high degree
of anaphylactic sensitivity to the autolysate derived from Type I
Pneumococcus. The "normal" antipneumococcic Type I rabbit
serum, from which the specific anticarbohydrate antibody had

been removed, also rendered guinea pigs anaphylactically hyper-sensitive to the A substance in the homologous autolysate. Before the removal of the anticarbohydrate antibody by precipitation *in vitro*, the serum sensitized guinea pigs to the type-specific soluble substance. After the antibody had been eliminated, animals injected with the serum failed to react to the injection of the carbohydrate, but responded typically when injected with the autolysate from virulent organisms of Type I. Furthermore, the animals showed no symptoms following the intravenous administration of autolysate derived from a rough strain of Type I or of autolysates of smooth strains of Types II and III.

The experiments in anaphylaxis showed that in the serum of rabbits receiving injections of formalinized pneumococci an antibody develops that reacts specifically with an antigen found in the autolytic products of Type I Pneumococcus. Similar autolysates of the other two types (II and III) of this organism, as well as rough strains derived from homologous or heterologous types, elicited no anaphylactic symptoms in guinea pigs. Under the same conditions, the nucleoprotein was likewise incapable of provoking specific shock. That the antigen in Type I autolysate responsible for the anaphylactic symptoms was not the specific carbohydrate appeared to Enders to be shown not only by the failure of the latter material, when pure, to produce shock in guinea pigs sensitized with anti-A rabbit serum, but also by the experiments with rabbit serum from which the carbohydrate antibody originally capable of sensitizing the animals had been removed, leaving unimpaired the power of the serum to sensitize the animals to the A substance.

The cellular carbohydrate from Type I Pneumococcus, likewise, caused immediate, lethal anaphylactic intoxication in guinea pigs previously injected with Type I antipneumococcic rabbit serum. Schiemann, Loewenthal, and Hackenthal[1231] reported a similar effect obtained with the carbohydrate preparation made by the method of Schiemann and Casper.[1228] These results, taken with further observations of Avery and Goebel (1931)[45] on the

immunological action of an antigen prepared by combining the capsular polysaccharide of Type III Pneumococcus with horse serum globulin, fully justify the conclusion that it is the soluble specific carbohydrate which determines the type-specificity of the hypersensitive state and of the anaphylactic reaction.

The contrasting differences in the sensitizing properties of serum from the immune rabbit and the immune horse were studied by Mehlman and Seegal (1934).[889] In conformity with the results of Avery and Tillett, guinea pigs injected parenterally with antipneumococcic rabbit serum were thrown into anaphylactic shock by the intravenous injection twenty-four hours later of the capsular carbohydrate of pneumococci corresponding in type to that of the immune serum. Guinea pigs similarly prepared with antipneumococcic horse serum were not susceptible to an otherwise shocking dose of the homologous antigen. Uteri removed from guinea pigs sensitized passively with the immune rabbit serum contracted characteristically on contact with the homologous specific carbohydrate, whereas uteri of guinea pigs injected with immune horse serum failed to react in this manner. Tests made to determine a possible anti-alexic activity of parenterally injected antipneumococcic rabbit and horse serum failed to show any marked difference between the two serums. In a second communication, Mehlman and Seegal[890] demonstrated in mice the same difference between the sensitizing property of antipneumococcic serum from the rabbit and from the horse. The sensitizing property of immune rabbit serum appeared to be independent of protective action, since the serum of both the immune rabbit and horse were equally effective in curing mice of infection with Pneumococcus.*

The comparative inability of antipneumococcic horse serum to sensitize the guinea pig passively to Pneumococcus is also evident when sensitivity is tested by intracutaneous injection of soluble specific substance. According to Mehlman and Seegal (1934),

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* Differences in the nature of antibodies in immune rabbit and horse serum have been discussed in Chapter XI.

preparations of Type I antipneumococcic serum, obtained from rabbits, sensitized the albino guinea pig so that an injection of 0.1 cubic centimeter of a 1 to 10,000 dilution of Type I polysaccharide into the skin of the ear elicited a positive reaction. However, when antipneumococcic serum from the horse was employed, the same dose of antigen gave variable results, although the injection of twice the amount evoked positive reactions. Experiments on the distribution of immune horse serum as compared with that of rabbit serum in the circulation and organs of passively immunized guinea pigs failed to disclose any explanation for the differences in the sensitizing action of the two serums.

Brown[155-6] reported similar observations on the ability of antipneumococcic rabbit serum to sensitize passively the guinea pig to the specific carbohydrate of Type I Pneumococcus. Brown found that the minimal amount of antiserum necessary to sensitize the guinea pig so that fatal shock followed the injection into the heart of the soluble specific substance was always greater than the quantity of serum required to sensitize to the cellular carbohydrate of Wadsworth. However, the minimal amount of each of the specific carbohydrates which induced fatal shock in guinea pigs previously sensitized by the injection of one cubic centimeter of immune rabbit serum was approximately the same.

The protective or inhibitory action of artificial, conjugated sugar-proteins on the anaphylactic reaction of guinea pigs passively sensitized with antipneumococcic rabbit serum, as reported by Tillett, Avery, and Goebel,[1407] was confirmed by Brown[156] in the case of the soluble specific substance and of the cellular carbohydrate. A subcutaneous injection of a suitable dose of soluble specific substance of Type I Pneumococcus given to guinea pigs twenty-four hours after passive sensitization with Type I antipneumococcic rabbit serum completely desensitized the animals to intracardial injection twenty-four hours later of capsular polysaccharide, but not to a similar injection of cellular carbohydrate. The latter substance, however, completely desensitized the guinea

pigs to the anaphylactic action of the soluble specific substance
and brought about partial desensitization to the cellular carbo-
hydrate. When capsular polysaccharide prepared according to the
original method of Heidelberger and Avery (probably the deacety-
lated carbohydrate) was added to the minimal dose of serum neces-
sary to sensitize, and the mixture injected at once into guinea
pigs, the animals failed to become hypersensitive to subsequent
intracardial injection of the same preparation, but acquired sensi-
tivity to the cellular carbohydrate. However, when cellular carbo-
hydrate was substituted for soluble specific substances in the ex-
periment, no sensitization to either capsular or cellular carbo-
hydrate could be demonstrated.

The results reported by Brown were in accord with observations
previously described by Wadsworth and Brown (1933).[1468] Spe-
cific immune serum, from which the precipitate, formed on the ad-
dition of SSS, had been removed, was still capable of sensitizing
guinea pigs to the cellular carbohydrate but not to soluble specific
substance. Antiserum, after precipitation with cellular carbohy-
drate, failed to sensitize the animals to either substance. The dif-
ference in the action of the two preparations of specific pneumo-
coccal polysaccharides was undoubtedly due to the lack of molecu-
lar completeness of the preparation of soluble specific substance
employed in the experiments.

RELATION OF PNEUMOCOCCAL ALLERGY TO PNEUMOCOCCAL IM-
    MUNITY

The experiments of Mackenzie[844] suggest a lack of correlation
between the hypersensitive and the immune states in guinea pigs
treated with Pneumococcus or its extracts. During the course of
immunity produced by the intraperitoneal injection of killed and
living broth cultures of virulent pneumococci, while the animals
showed high resistance to infection and possessed strong protec-
tive power in their serum, hypersensitiveness might or might not
be present. Mackenzie concluded that anaphylaxis to pneumo-

coccal protein was merely a concomitant of immunity without having any significant part in the immunological mechanism.

Sharp and Blake (1930)[1260] differed with the conclusions of Mackenzie and maintained that in rabbits a fairly close parallelism exists between cutaneous and pulmonary hypersensitiveness to pneumococcal autolysate, and that the inflammatory response of pulmonary tissue, resulting from contact with autolysate, depends on the allergic state of the animal rather than on inherently injurious substances in the autolysate. Sharp and Blake interpreted their observations as being in harmony with the theory that allergy may play a part in the pathogenesis of pneumococcal pneumonia in man.

Julianelle and Rhoades (1932),[696] in a study of the reaction of the lungs of rabbits to infection caused by intravenous injections of Pneumococcus, found that reactions occurred irregularly in the lung, and that in the lungs in which reactions did occur, the histological changes were no different in normal rabbits from the changes in rabbits made resistant by previous intravenous or intracutaneous injections of pneumococci. The authors concluded that the experiments afforded no evidence to support the view that the lesions in the lungs of rabbits, following the intravenous injection of pneumococci, were modified by any previous state of sensitivity.

Dermal Allergy

SKIN REACTIONS IN EXPERIMENTAL ANIMALS

The development of an allergic condition of the skin in guinea pigs following the intracutaneous injection of an alkaline extract of pneumococci occurred in experiments described by Mackenzie and Woo (1925).[845] About two-thirds of the animals so treated acquired cutaneous hypersensitivity but, upon the continuance of the injections, the capacity of the dermal tissues to react ceased. Neither the animals manifesting skin allergy nor those that failed to develop this type of hypersensitiveness showed any significant

alteration in susceptibility to pneumococcal infection by intra-
peritoneal inoculation. Similarly, animals desensitized by repeated
intracutaneous injection after the appearance of allergy ex-
hibited an unaltered susceptibility to infection.

In 1927, Zinsser and Grinnell[1581] investigated the action in the
skin of normal guinea pigs of various bacterial autolysates in-
cluding those prepared from pneumococci. Reactions were noted
only in the case of well-grown animals, whereas young guinea pigs
almost invariably gave a negative reaction on the first test. On
further study it was found that the cutaneous reactions were
manifestations of hypersensitiveness which could be elicited by
bile solutions of Pneumococcus as well as by similar autolysates.
Sensitization could be induced by the previous injection of dead,
intact bacteria, as well as of autolysates, but the actual skin re-
action was elicited only by autolysates. When properly treated,
insensitive animals could be rendered sensitive within a space of
five to seven days by the daily injection of autolysate. The sensi-
tivity thus produced was related to the bacterial species and not
to pneumococcal type. Using the rabbit, Julianelle (1930),[689]
after subjecting the animals to repeated intracutaneous injections
of heat-killed pneumococci, demonstrated increased skin activity
which reached a maximum after four to six injections had been
given and then diminished. After regression of the reaction to the
first injection of antigen into the skin, there frequently followed
a recrudescence or exacerbation of the reaction. By injecting the
serum of a highly reactive animal into a normal rabbit, the author
was unable to confer the property of skin-reactivity upon the re-
cipient.

In another paper, Julianelle[690] communicated further results ob-
tained in rabbits during a study of actively induced skin-sensi-
tivity to derivatives of Pneumococcus. Positive reactions were
evoked in animals that had previously received repeated intra-
cutaneous injections of heat-killed pneumococci, of the pneumo-
coccal nucleoprotein, and of a solution of the bacterial cell from

which the acid-precipitable and heat-coagulable proteins had been removed. The reaction appeared to be species-specific but not type-specific. A similar skin reaction to the protein of Pneumococcus occurred in rabbits following the repeated intracutaneous or intravenous administration of heat-killed organisms or their protein derivatives. The reactive function, although evidently related to the presence of humoral species-specific antibodies, occurred independently of resistance to infection.

A phenomenon related to skin sensitivity was the increased reactive power of the eye of rabbits immunized with heat-killed suspensions of smooth and rough pneumococci separately, as described by Julianelle.[691] Positive reactions could be elicited in immune animals by the instillation of nucleoprotein, or of a solution from which the acid-precipitable and heat-coagulable proteins had been removed. No reaction followed the similar application of living, rough cells or the protein-free, type-specific polysaccharide of Pneumococcus. Rabbits receiving intravenous injections of the intact cell or of the soluble derivatives of Pneumococcus failed to develop eye-reactivity, but acquired the property as a result of experimental infection with Pneumococcus. From the collected experimental evidence, Julianelle[692] concluded that the injection of heat-killed pneumococci into the skin created a special kind of skin and eye sensitiveness unrelated to the presence of circulating antibodies and not transferable from sensitive to normal rabbits.

In 1932, Julianelle with Morris,[693] by means of cutaneous and ophthalmic tests, disclosed another indication of a basic relation between pneumococci and streptococci and, in addition, a difference between the specificity of the reaction of the eye and of the skin of sensitized rabbits. Following repeated intracutaneous injections of heat-killed smooth and rough forms of Pneumococcus, rabbits acquired increased dermal sensitivity to both pneumococci and streptococci, while the injection of heat-killed indifferent streptococci produced skin sensitivity to both species of organisms. Injections of pneumococci into the skin were followed in

some rabbits by eye sensitivity to pneumococcal protein but not to streptococcal protein or to suspensions of either living organism, whereas similar injections of indifferent streptococci were followed, in some rabbits, by eye sensitivity to suspensions of living indifferent streptococci but not to suspensions of rough pneumococci or to nucleoprotein of either organism. Measured in terms of bacterial specificity, therefore, eye sensitivity appeared to possess a degree of specificity not shared by skin sensitivity. A collateral observation of Julianelle and Morris was to the effect that the serum of rabbits injected intracutaneously with pneumococci or indifferent streptococci contained agglutinins for both rough pneumococci and indifferent streptococci, but only those rabbits which received injections of Pneumococcus acquired resistance to pneumococcal infection.

Barnes* studied cutaneous reactions to pneumococcal protein and capsular polysaccharide in rabbits both actively and passively immunized against Pneumococcus and in horses undergoing active immunizing treatment for the production of antipneumococcic serum. In rabbits actively immunized with Type II vaccine, positive skin reactions were obtained following the intradermal injection of the homologous carbohydrate. The reaction consisted of an area of swelling and erythema having an average diameter of fifteen to twenty millimeters or more, and the crest of the swollen area was raised to a height of four to five millimeters. Positive reactions usually appeared within two hours after the injection as edematous areas which, within eighteen to twenty-four hours, became indurated and then persisted for more than two weeks. Similar injections into normal rabbits or rabbits immunized with heterologous strains of pneumococci were never followed by this type of reaction, although occasionally an early, transient erythema and slight swelling would appear, only to disappear within eighteen hours. Intradermal injections of somatic protein, in the amounts used, prepared from Type III pneumococci failed to

* Unpublished observations.

elicit reactions of significance either in normal rabbits or in rabbits previously immunized with killed pneumococci of the same type.

When a mixture of equal parts of 1 to 1,000 Type II polysaccharide and undiluted homologous immune rabbit serum was injected intradermally into normal rabbits, there developed a cutaneous reaction similar to that observed in actively immunized rabbits when similarly injected with the carbohydrate alone. In the few animals tested, the reaction following the antigen-antibody mixture was not so severe or so lasting as that evoked by the injection of polysaccharide into the skin of an actively immunized rabbit.

In another experiment the precipitin titer was determined on a sample of serum from a rabbit which had been actively immunized with Type II pneumococci and which exhibited a typical cutaneous reaction to the corresponding polysaccharide. Then, on the basis of body weight, a normal rabbit was given an intravenous injection of concentrated Type II antipneumococcic horse serum in an amount calculated to establish in the serum of the rabbit so treated a precipitin titer corresponding to that of the serum of the actively immunized animal. A precipitin test on serum from a test bleeding, taken one hour after the injection of immune serum, indicated that the desired result had been obtained. Skin tests with Type II polysaccharide performed at one-half-hour, one-hour, and two-hour intervals after the serum injection yielded reactions apparently identical with those observed when the same antigen was injected intradermally into rabbits actively immunized with Type II pneumococci. A repetition of the skin tests showed that positive reactions could be elicited for at least four days after the injection of serum. It was found, however, when normal horse serum was substituted for the specific, concentrated, immune horse serum, that no reaction took place.

In horses during the course of active immunization, positive cutaneous reactions could be obtained by the intradermal injec-

tion of homologous capsular polysaccharide. The reaction tended
to be larger in area but not so sharply defined as that in the ac-
tively immune rabbit. A peculiar phenomenon observed was the
disappearance and subsequent recurrence of the reaction in some
instances without intervening intradermal injections.* The long-
est period of time over which spontaneously recurring reactions
were observed was slightly more than six months. The injection of
pneumococcal protein into immune horses, and of type-specific
polysaccharide or protein into normal horses or into horses im-
munized with heterologous types of pneumococci, resulted in no
reaction. The experiments, though incomplete, point to a local
reaction in the dermal tissues between antigen and circulating
antibody or to a fixation of antigen by antibody in the dermal
cells rather than to a hypersensitive state of the cutaneous tissues.

Francis and Tillett (1931)[479] succeeded in demonstrating skin
reactions to capsular polysaccharides of pneumococci of Types I,
I, and III in rabbits actively or, under certain conditions, pas-
sively immunized to organisms of one or another of these types.
The reaction resembled that of Arthus, and was demonstrable only
when type-specific precipitins for the homologous polysaccharide
were present in the blood of the rabbit. A reaction was elicited in
84 per cent of actively immunized animals, the serum of which
contained type-specific precipitins, and a positive result was ob-
tained in all rabbits passively immunized with antipneumococcic
horse serum. Attempts to transfer the reactive principle from im-
mune rabbit to normal rabbit were unsuccessful. The recipients
in the latter group possessed no demonstrable circulating type-
specific precipitins. Francis and Tillett concluded that the reac-
tion produced by specific capsular carbohydrates is always asso-
ciated with a well-grounded type-specific immunity.

SKIN REACTIONS IN LOBAR PNEUMONIA

The value of skin reactions to tuberculin and to diphtheria

* Compare with observation of Julianelle, page 456, *ante.*

toxin in determining susceptibility or resistance to infection stimu-
lated interest in a similar application of pneumococcal antigens.
Weil (1916)[1507] employed a saline autolysate of Pneumococcus
(usually of Type I) and injected the material intracutaneously in
the scapular region. The injection was immediately followed at
times in pneumonia patients, and in normal subjects as well, by a
superficial, ill-defined, cutaneous blush. If the superficial erythema
faded within a few hours, the reaction was considered to be nega-
tive. If, however, within twenty-four hours there occurred a well-
circumscribed area of erythema, with infiltration and papular
elevation of the skin surrounding the point of puncture persisting
for twenty-four hours or more, the reaction was called positive.
During the course of pneumonia, Weil found the reaction to be
negative, but twenty-four hours to two weeks after crisis a con-
siderable proportion of patients reacted positively. Normal indi-
viduals and subjects suffering from diseases due to organisms
other than Pneumococcus might or might not exhibit a reaction.

Analogous results were reported by Steinfeld and Kolmer
(1917),[1315] who employed saline suspensions of washed organisms
of Types I, II, and III heated to 60°. A positive reaction consisted
in a definite papule with an area of erythema greater than one
centimeter in diameter accompanied by slight edema. Positive re-
actions were observed in 30 per cent of patients suffering from
lobar pneumonia, but consistently negative results were obtained
with normal individuals and with those afflicted with various
chronic diseases. All positive reactions noted occurred after crisis
or after infection had persisted for a long period of time. Stein-
feld and Kolmer stated that there was no constant relation be-
tween reactions to the protein of the various types of pneumococci
used and the types found in the sputum. Weiss and Kolmer
(1918)[1510] employed as antigen hemolytic toxin prepared by dis-
solving washed, living pneumococci in sodium choleate. Among
adult patients with lobar pneumonia the intracutaneous reaction
was elicited as early as the fifth and as late as the thirteenth day

of the disease (two days before and six days after crisis). In children the reaction was demonstrable at about the same time, but was negative immediately following crisis or within one or two days after crisis. When the pneumonia resolved by lysis, the patients reacted as late as the thirty-second day. In general, the test was positive in all active cases but was negative in all control patients whether children or adults. Weiss and Kolmer considered that the phenomenon was a manifestation of a state of allergy to toxin but, since the reactivity of the skin was present during the stage of toxemia and disappeared during convalescence, it is difficult to look upon the reaction as being strictly allergic in nature, at least in so far as toxin is concerned.

Bigelow (1922),[115] employing saline and aqueous autolysates of pneumococci as antigens, succeeded in evoking type-specific dermal reactions in some cases, while in others and in normal controls a reaction occurred differing in character from that resulting when the type of autolysate and of the infecting organism were homologous. Larson (1926)[787] also reported irregular results of skin tests obtained in normal persons and pneumonia patients injected with pneumococcal filtrates. He apparently assumed that the reaction was due to toxin since allergy was not mentioned in the communication.

The question became somewhat more confused by the observations of Herrold and Traut (1927).[637] The majority of pneumonia patients reacted negatively to filtrates of broth cultures of Pneumococcus, whereas nearly all normal individuals similarly tested also gave negative reactions. By repeated tests on pneumonia patients it was found that the condition preventing a reaction developed early in the disease and usually persisted throughout convalescence. The reactive ability of the individual corresponded to the capacity of the serum to neutralize the active principle of the filtrate, the serum of a non-reactive subject usually effecting a partial or complete inhibition of the antigen.

Comparable to the results of Herrold and Traut were those of

Kramár and Gyüre (1929).[754] Injecting intradermally suspensions of pneumococci of the different serological types, the authors noted positive skin reactions in only 18 per cent of pneumonia patients but in 50 per cent of healthy individuals. In the case of the pneumonia patients reactivity of the skin appeared in eight to sixty days after resolution of the pneumonic process. The reaction was, in general, type-specific for Types I, II, and III, as judged by the serological relation of the antigen employed to the organism isolated from the sputum of the subject tested.

Contradictory results were described by Tillett and Francis (1929),[1408] who found that pneumococcal polysaccharides, when injected intradermally into patients convalescent from lobar pneumonia, were capable of eliciting a definite response. A patient's ability to react was apparently associated with both recovery from infection and the presence of type-specific antibodies in the circulating blood. In addition to the action of the polysaccharide, the so-called nucleoprotein of Pneumococcus, when similarly injected, caused a local cutaneous reaction in subjects during convalescence from pneumonia. The local lesion resulting from the injection of protein was tuberculin-like in character, and differed from that evoked by type-specific polysaccharides in gross appearance, time of development, and duration. Although the presence of precipitins reactive with pneumococcal protein could be demonstrated in individuals acutely ill with or convalescing from pneumococcal pneumonia, the concentration of antipneumococcic antibodies in the blood serum appeared to be unrelated to the patients' capacity to react to intradermal injection of the protein.

In a second communication, Francis and Tillett[478] amplified their observations on the appearance of cutaneous reactions during lobar pneumonia. Immediate wheal formation and erythema followed the intradermal injection at the time of crisis of homologous polysaccharide in 100 per cent of Type I patients, in 55.8 per cent of those infected with Type II Pneumococcus, and in 44 per cent of those infected with pneumococci of Type III. A de-

layed reaction ensued after a similar injection of pneumococcal protein. The appearance of circulating antibodies for one or more heterologous types in a group of patients repeatedly tested with soluble specific substance was believed by the authors to be referable to the antigenic action of the polysaccharide introduced into the skin during successive tests.

In opposition to the conception that skin reactivity to the capsular polysaccharide of Pneumococcus is associated solely with recovery from pneumonia are the results of Alston and Lowdon (1933).[10] The majority of the individuals studied (63 per cent) who had not recently suffered from pneumococcal infection reacted positively to the intradermal injection of Type II polysaccharide, the percentage of reactions increasing in the older age groups. Retested five to twelve months later, 92 per cent of the subjects previously exhibiting a positive reaction were found to have retained the ability to react to the same antigen. The capacity to respond to similar injections decreased and in some cases entirely disappeared when the test injections were repeated at short intervals. The authors also reported that a considerable number of normal subjects, especially older persons, exhibited a delayed reaction in addition to the immediate reaction or independent of it.

The manifestation of both immediate and delayed reactions may be explained by the fact that the introduction of pure pneumococcal capsular polysaccharide into the skin of a positively reacting subject is promptly followed by a local inflammatory condition, while after the similar injection of the C Fraction or of somatic protein, an interval elapses before any local reaction occurs. Skin sensitivity to pneumococcal type-specific carbohydrates and the presence of specific antibodies in the circulating blood were found to exist independently of each other. The findings of Alston and Lowdon agree with those reported in the previous year by Sutliff and Finland,[1360] who reported that skin reactions to acetic acid-precipitable protein and autolysates of Pneumococcus were nega-

tive or rarely positive in infants, infrequently positive in child-
hood, and positive in a high percentage of adults.

Finland and Dowling (1935)[442] performed skin tests on pa-
tients recovering from lobar pneumonia and also on normal indi-
viduals and persons suffering from non-pneumococcal affections.
The antigens employed were the capsular polysaccharides of pneu-
mococci of Types I, II, and III, and the cellular carbohydrate of
Wadsworth and Brown. Immediate cutaneous reactions, similar
to those described by Tillett and Francis, were elicited in patients
recovering from pneumonia. These reactions were, in general,
type-specific and were associated with the homologous antibody in
the serum. Similar reactions were obtained with preparations of
soluble specific substance and cellular carbohydrate prepared from
the same pneumococcal types by the methods of Heidelberger,
Goebel, and Avery and those of Wadsworth and Brown, respec-
tively.

Characteristic delayed reactions were seen only with the cellu-
lar carbohydrate and were not associated with type-specific anti-
bodies. The delayed reactions were most frequently observed fol-
lowing the injection of the cellular carbohydrate of the atypical
Type I Pneumococcus and of the analogous antigen prepared
from the virulent Type I strain. Delayed cutaneous reactions to
the cellular carbohydrate of an atypical Type I Pneumococcus
were obtained regularly during the febrile stage of a variety of
infectious diseases but could not be evoked soon after recovery in
these cases. The results are similar to those obtained by Francis
and Abernethy[475] with the somatic C Fraction of Tillett and
Francis.

Among the non-pneumonic subjects on whom observations were
made, immediate reactions in general were uncommon. They oc-
curred most frequently with the cellular carbohydrates of Type I
and atypical Type I pneumococci. Delayed reactions with soluble
specific substance of any type did not occur, but they were fre-

quent with the cellular carbohydrates of Type I and atypical Type I organisms.

In the group of subjects who gave a previous history of pneumonia, none of the eight tests done with capsular polysaccharide was positive. A higher incidence of positive, delayed reactions was, however, obtained in the small number of subjects of this group tested with the cellular carbohydrates.

The lack of uniformity in specific cutaneous reactions occurring in normal human beings and in those ill with or convalescing from lobar pneumonia was encountered by Cecil and Austin (1918)[204] in following the administration of vaccines made from pneumococci of Types I, II, and III and used for prophylactic injection of healthy men. Small, sterile infiltrations, disappearing spontaneously, occasionally followed the injection of vaccine, and similar lesions appeared in the same individuals after subsequent injections. Subjects exhibiting these reactions rarely possessed any notable amount of agglutinative or protective antibodies in the serum after vaccination, nor was the reactive property specific for any type of Pneumococcus.

Gutfeld and Nassau (1926)[580] treated nurslings ill with bronchopneumonia with vaccines composed of heat-killed pneumococci, and injected normal children or those ill from some cause other than bronchopneumonia intracutaneously with vaccine or with watery autolysates of the same cultures from which the vaccines were prepared. The development of a reddened papule was observed at the site of puncture, appearing within eighteen or twenty-four hours and disappearing after forty-eight hours. No child under one and one-half years of age gave a positive reaction, nor did any of the young patients suffering from bronchopneumonia. The reactions occurred in more than one-half of the normal individuals tested and in a like proportion of those not afflicted with the disease. When the autolysate was mixed with serum

of non-sensitive children no reaction took place, whereas the serum of sensitive children failed to neutralize or inhibit the action of the antigen. Gutfeld and Nassau assumed that individuals failing to react to the intradermal test possessed in the skin or in the serum a specific antibody similar to antitoxin, inherited in the case of young nurslings or developed during an attack of bronchopneumonia and then later lost.

Jamieson and Powell (1931)[676] believed that pneumococci produce toxic substances not unlike those obtained from various streptococci. The substances could be detected and measured by skin tests on human subjects and by similar tests performed on rabbits of a suitable breed. Serum yielded by horses that had been treated by subcutaneous injections of the toxic substance possessed the ability to neutralize the skin-reactive property of the toxic antigen. In a second paper, Jamieson and Powell reported the development of positive reactions when filtrates of pneumococci of Types I, II, III, and IV were injected intradermally into large rabbits. Similar reactions to filtrates of all four pneumococcal types were also observed in the case of twelve normal human subjects, who exhibited a degree of sensitivity approximately one hundred times that shown by rabbits. The toxic filtrates could be neutralized by the special immune serum developed in horses by the injection of the same antigen.

The work of Coca[245] bears certain resemblances to that of Jamieson and Powell.[676] Filtrates of pneumococci exhibiting toxic action for young children when injected subcutaneously also induced local inflammatory reactions in the skin at the site of puncture. In Coca's first experiments the filtrate was introduced into the child's skin and at the same time a considerably larger dose of the filtrate was injected subcutaneously. Subsequent skin tests performed with the filtrate on the children so treated were attended by a diminished reaction. Treatment of the subjects with a toxoid prepared by heating the filtrates with formalin tended to reduce the number of positive reactions. When applied to adults giving no

history of previous pneumonia, the test was positive in 56 per cent of the subjects tested. In the case of individuals having a history of pneumonia in the past, 15 per cent of the subjects reacted, while in convalescent patients only 5 per cent exhibited positive reactions. Coca reported that not all filtrates tested were antigenic and, at the time, ventured no statement concerning the type-specificity of the phenomena.

Recently Coca,[246] after a further study of the alleged pneumococcal toxin, stated that the action of the toxin in the skin was type-specific and, furthermore, that the toxin was not the type-specific polysaccharide.

The application of the skin test as a guide to serum therapy and as a prognostic aid was suggested by Francis (1933).[473] All but one of the patients who had recovered from Type I Pneumococcus pneumonia (the great majority of them had received specific antipneumococcic serum) gave an immediate reaction to the Type I polysaccharide at about the time of recovery. Francis claimed:

The test has distinct advantages over the agglutination reaction in that it is not merely an index of circulating antibodies. When positive, it invariably denotes that recovery has begun; when negative, it indicates further serum therapy. The mechanism of the positive skin test is closely related to that operative in recovery from pneumonia, and is apparently the resultant of antibody and tissue activity.

THE MECHANISM OF DERMAL ALLERGY

In addition to the foregoing discussion, two studies may be mentioned which have a direct bearing on the physiological or immunological processes operating in the production of the allergic state and in the manifestations of the hypersensitive condition as elicited by pneumococcal derivatives. Martin and Hill (1930)[866] tested the effect of an alien protein on the reactivity of dermal tissues in albino guinea pigs receiving intradermal injections of living pneumococci. One group of animals received into the skin of

the left flank an injection of a suspension of washed pneumococci followed immediately by a similar injection of milk protein on the right flank. Forty-eight hours later they were given on the left side at some distance from the first puncture an injection of the same organism heated for thirty minutes at 60° and again milk protein into the skin on the right flank. The second group of guinea pigs were treated in the same manner but without the injection of milk protein. Examination of the animals of the second group revealed an area of induration and inflammation at the site of the injection of pneumococci, whereas the animals that had received milk protein in addition to the pneumococcal suspension showed only the slightest response to the bacterial antigen and none to milk protein. When intramuscular injections of milk protein were substituted for intradermal administration, with all other conditions of the experiment the same as before, the reactions of the animals so treated were less pronounced than those in the guinea pigs receiving only pneumococci, but more marked than those occurring when the protein was injected into the skin. While it is apparent that the injection of foreign protein inhibited the reactivity of the cutaneous tissues to the injected suspension of heated pneumococci, the significance of the experiments is not clear.

Kramár and Gyüre (1930)[756] sought other means for learning the manner in which the allergic manifestations of the skin take place. A comparison was made of the bactericidal, bacteriotropic, protective, and antitoxic action of the serum, plasma, and defibrinated blood of human beings who reacted either positively or negatively to an autolysate of Type II Pneumococcus. From the experiments there appeared to be no correlation between the content of humoral antibodies and the outcome of the skin tests. The authors then studied the relation of the skin reactions in man evoked by pneumococcal autolysate with the so-called "pharmacodynamic reaction" of Hecht and von Groer, which consisted in determining the maximal dose of epinephrin and caffein-sodium salicylate which, when injected alone into the skin, would produce

twenty-four hours later no redness at the point of injection. For the details of the experiment the original communication of Kramár and Gyüre should be consulted. The results led Kramár and Gyüre to conclude that the cutaneous reaction to pneumococcal autolysate was similar to that produced by tuberculin and the opposite of that caused by diphtheria toxin and, therefore, that the pneumococcal skin test was an allergic reaction and was an expression of cellular immunity.

In a theoretical discussion of the mechanism of skin reactivity to Pneumococcus, Kramár and Gyüre presented the theory that allergy represents a labile condition of the animal organism, since artificial immunization causes positive reactivity to become negative reactivity, while colds, exhaustion, fatigue, massive infections, and diseases other than pneumonia, and possibly the age of the individual, change the state of allergy to one of anergy. Kramár and Gyüre presented the following schematic representation of the factors concerned in the three hypothetical states prevailing in man in relation to hypersensitivity to Pneumococcus.

I	II	III
Anergic phase—no protection	*Allergic phase—labile immunity*	*Fastness—stable immunity*
Skin test—negative	Skin test—positive	Skin test—negative
New born subjects	The majority of children and adults	The minority of children and adults
The number of negative reactors decreases with age	The number of subjects increases with age among nurslings	Those artificially immunized

The authors explain that shifts in phase to the right are influenced by increasing age of infants, by recovery from pneumococcal infection, and by repeated slight infections such as common catarrh of the upper respiratory passages. Reverse shifts to the left are brought about by the debilitating causes already mentioned.

The foregoing presentation of the subject of dermal allergy to

pneumococcal products is bewildering, and from the data as pre-
sented it is difficult to gain a clear conception of the true signifi-
cance of specific skin tests in health, during pneumococcal infec-
tion, and in pneumonia patients receiving specific serum therapy.
The primary purpose of the authors of the present volume has
been to recite the many observations as they have been reported in
the literature in order that no contributions, whatever might be
their value, should be neglected. It should be borne in mind that in
many of the reports abstracted the materials employed for testing
skin reactivity were frequently mixtures of the native constituents
in more or less altered form of Pneumococcus, and of autolytic or
metabolic products of the cell. It is not surprising therefore that
the skin both of healthy persons and of pneumonic patients should
display a diversity of reactive manifestations following the intra-
dermal injection of bacterial suspensions in different stages of
autolysis, or of extracts or filtrates of pneumococci. Observations
of this nature, having been cited, may now be partly disregarded
and the discussion confined to the action of the more tangible, iso-
lated components of Pneumococcus.

To arrive at the true significance of cutaneous reactions in hu-
man beings following the intradermal introduction of pneumococ-
cal materials it is necessary to consider the action of the separate
constituents of the pneumococcal cell employed in a comparatively
pure state and to determine as far as possible the experience of the
subject tested in regard to past or present pneumococcal infection.

With improved methods in the preparation of bacterial protein
and somatic and capsular polysaccharides, antigens are now avail-
able for an analytical study of skin tests, but it is not always pos-
sible to ascertain the immunological condition in respect to Pneu-
mococcus of the person to be tested.

A positive cutaneous reaction to the protein fraction of Pneu-
mococcus may be elicited by the appropriate injection of the anti-
gen into the skin of individuals who give no history of pneumonia.
In patients ill with the disease a similar injection gives a negative

result during the acute stages of the disease but a positive reaction appears late in the period of convalescence (Abernethy and Francis[2]). The reaction in this case is of the delayed variety, not appearing until several hours after the injection is made, and immunologically is species-specific and not type-specific.

When the somatic C polysaccharide of Pneumococcus is employed, a positive cutaneous reaction may be obtained in the great majority of patients during the acute stages of the disease and has been demonstrated as early as twelve hours after the initial chill. The state of reactivity to the C Fraction usually persists throughout the acute febrile period. With the onset of recovery, whether associated with crisis or lysis, the reaction decreases markedly in intensity and, during an uncomplicated convalescence, in the majority of instances can no longer be demonstrated. Reactivity may persist or reappear if complications develop. If the case terminates fatally, the patient may fail to give a skin reaction to the somatic carbohydrate during some stage of the acute disease (Abernethy and Francis). The local manifestations appearing after the intradermal injection of the C Fraction occur in a positively reacting person within fifteen to twenty minutes and in their early development resemble those of the capsular polysaccharide skin test, but the zone of erythema is less intense and pseudopods extending out from the central wheal, so frequently seen in the latter, are rarely encountered. Within an hour the acute phase has usually passed and is then followed by a delayed reaction, an edematous erythema. The delayed reaction begins to appear in two to three hours, is well marked in six to ten hours, persists for eighteen to twenty-four hours, and then fades, leaving a residual brown stain (Abernethy and Francis). The C polysaccharide, being common to all pneumococci, is heterogenetic in its capacity to elicit a positive reaction in the skin of pneumonia patients and, furthermore, cutaneous reactivity to this carbohydrate component of pneumococci is not limited to patients infected with

organisms of that bacterial species, since a positive reaction has been obtained in patients ill with other infectious febrile diseases.

The capsular polysaccharides of Pneumococcus, on the other hand, when injected intradermally into pneumonia patients are highly type-specific in their action, and by their action serve as an indicator of the immunological state of the individual tested. When positive, the reaction is immediate, with wheal formation and the development of erythema. Differing from the conditions observed when pneumococcal protein or somatic carbohydrate are used as test agents, reactivity of the dermal tissues is nearly always absent in normal human beings and in patients during the early stages of pneumonic infection. As the disease progresses, usually shortly before, during, or after crisis, the skin of the patient acquires the capacity to react to the capsular polysaccharide and the condition may persist for a shorter or longer time during and after convalescence. A positive reaction is regarded as evidence of definite immunological response on the part of the patient to the infection. A positive reaction denotes that recovery is taking place; a negative reaction is interpreted as indicating that the defensive forces of the body, whether natural or artificially aroused, are unequal to cope with the infection. Intradermal tests with type-specific polysaccharides, therefore, may be applied to estimate the adequacy of serum treatment.

The percentage of positive cutaneous reactions elicited by the injection of homologous capsular polysaccharide varies with the serological type of Pneumococcus responsible for the infection. Almost all patients suffering from Type I infections react to the Type I polysaccharide; approximately slightly more than one-half the number of patients with Type II disease respond to the intradermal injection of Type II polysaccharide; and in Type III infections the percentage of positive reactors is even smaller. As has been mentioned earlier in the text, the intradermal injection of pneumococcal polysaccharides into human beings may induce sub-

sequent reactivity of the dermal cells, and this fact is to be borne in mind in studies on skin sensitivity where repeated intradermal injections of these agents are involved.

The true nature of the phenomenon of skin reactivity to Pneumococcus or its components still remains hidden. The resulting skin activity to capsular polysaccharide following the administration of homologous type-specific antipneumococcic serum has led to the hypothesis that the specific reaction is to be explained on the basis of local interaction of antigen and circulating antibody in the dermal tissues but the hypothesis fails to account for the repeated observation that a negative cutaneous reaction may be obtained even when the blood of the subject contains demonstrable antibodies. There is, to be sure, a certain parallelism between the results of serological tests and skin tests in pneumococcal pneumonia with the somatic carbohydrate C, but the correlation does not necessarily hold when the capsular polysaccharide is used as the test material.

The claim that pneumococcal toxins and their homologous antitoxins operate in this immunological reaction requires further investigation before it can be accepted. The participation of humoral antibody and the possible reactivity of the dermal cells induced by pneumococcal infections are questions shrouded in obscurity, and it would be presumptuous at the present time to venture any more definite statements regarding this interesting and important phenomenon.

THE SHWARTZMAN PHENOMENON

In 1928, Shwartzman[1263] described a dermal reaction to bacterial extracts in rabbits, which consisted in a severe local activation, by intravenous injection of the extract, of a previously inert intradermal injection of the same material. The phenomenon—since called the Shwartzman phenomenon—appeared to differ from the usual toxic or allergic cutaneous reactions in the following fea-

tures: local reactivity; the short incubation period necessary to induce the local reactivity; the short duration of the state of reactivity; the ability to induce local reactivity by a single skin injection; the severity of the reaction; and the necessity of giving the second injection of the toxic agent by the intravenous route.

The phenomenon elicited by the injection of pneumococcal materials was first reported by Cope and Howell[277] in 1931. A substance obtained by dissolving pneumococci in bile evoked a local skin reaction when its intradermal injection was followed by a subsequent intravenous injection into rabbits. The reaction could not be obtained by the use of pneumococcal filtrates prepared by methods in which the cell body was not disintegrated. Type-specificity of the reaction was demonstrated in 44.4 per cent of the experiments, but positive reactions were observed in 13.8 per cent of the experiments in which heterogeneous types of pneumococci were employed for the intradermal and intravenous injections.

A wide deviation from type-specificity and even from species-specificity in the mechanism of the phenomenon was later shown by Shwartzman.[1264] Culture filtrates of Type III Pneumococcus and agar washings of *B. typhosus* were simultaneously injected at separate sites into the skin of the ear of rabbits. No reaction followed this preparatory injection. When the pneumococcal filtrates were injected intravenously twenty-four hours later, no activation at the site of the pneumococcal extract injection occurred, but in the majority of the animals a marked reaction was observed at the point where the preparatory injection of washings of the typhoid bacilli had been made. From the experiments, Shwartzman concluded that pneumococcal filtrates were devoid of the preparatory principle but were potent in reactive factors for *B. typhosus* agar-washings if not for the pneumococcal substance. There was another factor in the phenomenon, as observed under the experimental conditions, and that was the ability of pneumococcal extracts, that had lost their reactivating power for preparatory injections

of *B. typhosus* agar-washings, to regain potency after being mixed with (type?) specific horse antiserum and to a lesser degree with type-specific rabbit antiserum.

In a subsequent paper, Shwartzman[1265] reported additional observations on the ability of homologous antigen-antibody combinations introduced into rabbits intravenously to render tissues vulnerable to the action of soluble products of heterologous bacterial species previously injected into the skin of the rabbit ear.

The work of Cope and Howell points to the species-specificity and, to a certain extent, to the type-specificity of the phenomenon. In following through the several communications of Shwartzman, the conviction is formed that there is no specific immunological relation between the action of the preparatory principle and that of the reactivating substance. The conditions of the experiments as conducted by Shwartzman give the impression that Pneumococcus contains no substance preparatory or activating to itself, although from the organism a substance may be obtained that causes dermal tissues to become susceptible to the local action of material present in typhoid agar-washings. It would be out of place here to enter into any detailed discussion of the factors involved in the operation of the Shwartzman phenomenon. However, from the facts available, it is difficult at present to appraise its significance in the pathology and immunology of pneumococcal infections.

Summary

Nature endows some animals with means for defending themselves against infection by pneumococci. The integument of the body and of its passages and cavities presents obstacles to the entrance of the bacteria into the deeper tissues. When, however, entrance is effected, the cocci encounter in the blood stream antagonistic forces. Among the forces may be a body temperature inimical to the proliferation of the cocci—the naturally high temperature in the case of birds, or the febrile reaction in rabbits

engendered by some strains of pneumococci, varying with the species and age of the animal host. There are also normal opsonins, which prepare the invading bacteria for ingestion by the normal phagocytic cells. In addition to the opsonins protective substances are present in the serum of normal animals to ward off the attack, but these natural protective antibodies may be effective for one or more but not for all serological types of Pneumococcus. The possession or lack of these secondary, humoral defensive antibodies appears to parallel the natural susceptibility or resistance of different species of animals to pneumococcal infection. Within the species, new-born members are devoid of these circulating antibodies, but with increase in age there is a corresponding augmentation of these factors.

During pneumococcal infections in man, when native resistance is overcome, the body cells, aroused to activity by the stimulating components of the bacteria, elaborate motile immune substances, which comprise agglutinins, precipitins, opsonins, and protective antibodies. A substance, as yet of unknown nature, appears early in the serum of the pneumonia patient, which is capable of precipitating the C carbohydrate of Pneumococcus, but the true specific immune substances develop as the disease progresses, usually appearing in greatest quantity at the time of crisis, persisting in the blood stream for a while, and then disappearing during or after convalescence. The humoral antibodies thus evoked are specific for the serological type of the infecting strain, but it is conceivable that the recovered patient may possess for a time some residual immunity to organisms of a type other than that of the infecting pneumococcus. The presence of specific humoral antibodies does not always spell recovery for the patient, but their absence usually presages death. From the pneumococcal cell, as a result of its disintegration in the body, polysaccharides are introduced into the blood and, when in sufficient concentration, inhibit or block the action of the antibodies.

The humoral effects and cellular reactions taking place during

pneumococcal infection may be artificially reproduced in suitable normal animals by the judicious parenteral introduction of pneumococci or of their antigenic constituents. The properties of the serum of artificially immunized animals may be transferred passively to normal animals. Owing to differences in the nature of the immune substances in the serum of animals of diverse species, the successful transference of passive immunity depends on the animal selected as the source of the immune serum.

During the development of immunity, whether evoked in a natural way or by artificial means, the tissue cells acquire a heightened sensitivity to Pneumococcus and some of its derivatives. Hypersensitivity in some animals may be manifested by an acute and fatal anaphylactic reaction, by increased contractility of the uterus, and by the inflammatory reaction of the cutaneous and pulmonary tissues arising upon the appropriate introduction of pneumococcal antigen.

The induction of the hypersensitive state and elicitation of the characteristic manifestations depend on the nature of the antigen employed. It is the capsular polysaccharide of the pneumococcal cell that orients the sensitizing stimulus and that is responsible for the type-specificity of the visible phenomena. In the case of hypersensitiveness to Pneumococcus, the allergic condition may be suspended and the specific reactions prevented temporarily by the introduction into the body of the polysaccharide homologous with that of the antigen used to induce allergy.

PNEUMOCOCCAL VACCINES

The preparation of vaccines from pneumococci, their components, or derivatives; the use of vaccines in the prevention and cure of pneumococcal infections; with a discussion of the theoretical and practical factors involved.

THE very nature of pneumococcal disease would seem to preclude the possibility of engendering by artificial means any secure or lasting protection against invasion of the body by pneumococci. The sudden onset, the often brief and stormy course, and the abrupt termination of the disease, so dramatically exemplified in lobar pneumonia in man, constitute a syndrome which results only in a fleeting insurance against a second attack by the same bacterial species. In diseases such as smallpox and anterior poliomyelitis, the morbid processes are caused by toxins or by living viruses, and specific stimulation of the immune mechanism usually eventuates in lasting defense. In infections by Pneumococcus, however, the struggle is between the natural forces of the body and the living cocci, and if the issue is successful on the part of the animal, the body is left almost as defenseless against these same bacteria as it was before the conflict.

Therefore, it is unreasonable to expect that the administration of pneumococci or of pneumococcal derivatives, no matter how artfully modified so as to be no longer capable of harm, would confer upon the recipient more than a short period of freedom from subsequent invasion by Pneumococcus. The attempts of immunologists to devise a suitable immunizing agent have been notable more for their ingenuity than for their success in establishing an immune state of high degree or of long duration. To be sure, there is abundant experimental evidence, as has been presented in previous chapters, that suitable antigens prepared from pneumococci and

introduced into the animal body stimulate the production of specific antibodies in even less time than do true toxins or filtrable viruses; but while these specific antibodies may guard the individual against infection by Pneumococcus, the same evidence shows that the immunity so induced, when at all effective, is specific only for the serological type of the antigen employed and at best is disappointingly transient.

In the chapters on antigenicity (X), antibodies (XI), and host response to antigenic action of Pneumococcus (XII), the facts presented would seem to warrant the conclusion that antigens representing the components of the pneumococcal cell as they exist in the cell at the height of vital function are, as a rule, the most effective in establishing the immune state. With Pneumococcus as with other bacterial material, attempts by means of physical or chemical agents to rob the antigen of undesirable properties or to isolate or concentrate the antigenic principles frequently so disrupt or denature the constituents of the cell as to deprive the agent of its immunizing properties. However, the protection of the individual or of groups of individuals against pneumococcal infection is often so desirable or so urgent that, bearing in mind the limitations of the practice, the administration of vaccinal agents made from pneumococci is fully justified. The complications presented by the type-specific action of pneumococcal antigens, changes in the prevalent variety of serological types, and the temporary nature of the protection conferred only spur the immunologist to further endeavor to increase the effectiveness of pneumococcal vaccines.

Basically the antigens employed for the production of active immunity in experimental animals and man fall into four general groups.

1. Saline suspensions of pneumococci devitalized by heat, formalin, phenol, soaps, or other physical or chemical agents. Vaccines of this kind may be assumed to represent all the constituents of the cell in their natural state, making allowance for such alterations in intrinsic

properties as may have occurred in the process of preparation. In some preparations the organisms are sensitized by appropriate treatment with homologous immune serum.

2. Extracts of pneumococci made in water, saline solutions, bile or bile salts, and other solvents, freed from cellular elements and containing the substances of the bacterial cell soluble in these solutions. In the preparation of extracts, autolysis of the cocci may or may not have occurred.

3. Autolysates of pneumococci taken at different stages of self-digestion of the coccal bodies and usually cleared of formed cellular material by Berkefeld filtration or some other method for removing particulate matter.

4. Solutions of the various chemical components of the pneumococcal cell isolated from supposedly non-antigenic substances. The solutions may be composed of only one constituent or of two or more substances derived from the cell. In the case of these solutions the question should be borne in mind how closely the ingredients approach the antigens as they exist preformed in the living and fully functioning virulent bacterial body or, conversely, to what extent their molecular structure has been changed by the manipulations required in manufacture.

In the present chapter attention will be directed chiefly to the preparation and the immunizing effect of those vaccines that have been employed for prophylaxis or therapy in man. The rationale of the use of pneumococcal vaccines is based on two kinds of evidence: the result of prophylactic injection in experimental animals, and the effect of vaccines in the prevention and cure of specific infection in human beings.

EXPERIMENTS ON MONKEYS

The previous chapters of this volume are replete with references to the capacity of Pneumococcus or its derivatives to induce in mice, rabbits, and other domestic animals the formation of specific antibodies contributing to the immune state. The earlier experiences in which the monkey was the test animal, because of the closer zoological relationship of that species to man, are perhaps the most significant in estimating the probable effect of the appli-

cation of the same biological agents in the case of human beings. Cecil,[202] with his associates, Blake and Steffen,[206, 212, 214-5] in a series of studies on the immunization of monkeys with different pneumococcal vaccines administered by various routes, learnt that because of the greater susceptibility of the monkey to pneumococci as compared to man, the test animals failed to respond as readily to the same amounts of vaccine. In order to induce a degree of active immunity sufficient to protect monkeys against experimental pneumonia incited with the homologous type of organism, it was necessary to employ small doses of living, virulent pneumococci, or to treat the animals by three intratracheal implantations of pneumococcal vaccines (Cecil and Steffen[214-5]). The subcutaneous injection of saline suspensions of killed pneumococci induced a greater amount of protective substances in the vaccinated animals than did lipovaccine, but in both cases the appearance of specific humoral antibodies was not always accompanied by protection against subsequent infection. By the subcutaneous, intravenous, and intratracheal route, three spaced injections of large doses of saline vaccine were required to immunize the animals against inoculation with homologous pneumococci of the four different types employed. When the immunizing treatment was carried out by way of the trachea, the immunity induced appeared to Cecil to be cellular rather than humoral in character. Large doses were required, no matter by which route given, while intravenous injection of the vaccine resulted in a higher degree of immunity than administration by the other methods tried.

Cecil concluded that immunity to Pneumococcus, like other forms of immunity, is relative and depends on the capacity of the individual to elaborate antibodies, on the virulence of the invading strain, and on the size of the dose of culture experimentally injected, to which might be added the virulence of the culture from which the vaccine is prepared, the amount, the number of injections, and the site selected for the injection of vaccine. Granting that, because of the greater susceptibility of the monkey to pneu-

mococcal infection, the analogy between effects in the monkey and man is not a strict one, Cecil's success in protecting the more highly susceptible animal against invasion by Pneumococcus pointed to even greater possibilities of the prophylactic use of pneumococcal vaccines in man.*

TYPES OF VACCINES EMPLOYED

Saline suspensions of heat-killed pneumococci have been used by Wright, Morgan, Colebrook and Dodgson,[1543-4] Cecil and his colleagues, by Lister,[816] Maynard,[872] and others; similar suspensions mixed with heat-killed bacteria of other species by Von Sholly and Park;[1452] suspensions of the organisms in oil by Russell,[1197] Rosenow and Sturdivant,[1173] Howell,[661] McCoy, Hasseltine, Wadsworth and Kirkbride;[875] formalinized suspensions by Ferguson;[436] pneumococci devitalized by tricresol, by phenol (Levy and Aoki[801]), and by hydrochloric acid by Kolmer and Rule;[747] soaped cultures by Olson[1029-30] and by Larson;[789] cultures killed by Merthiolate by Sutton, Kendall, and Rosenblum;[1366] washings of agar cultures—the so-called "Immunogen"—by Ferry and Fisher,[438] and by Horder and Ferry;[655] extracts of pneumococci and filtrates of broth cultures by Barach;[76] autolysates by Rosenow[1171] and others; pancreatic digestion products of pneumococci by Hirschfelder;[648] neutralized alkaline extracts of pneumococci by Brotzu;[150] while Ziegler[1573] employed a hypothetical substance obtained by lysis of pneumococci with sodium taurocholate and named by him "Pneumocholin." In these later days, purified constituents of the pneumococcal cell are being studied for their immunizing action on large groups of men by Felton.

DOSAGE

Wright *et al.*,[1545-6] in 1914, gave subcutaneous doses of from 250 million to 1,000 million cocci; amounts in excess of the latter count

* The efficacy of vaccination in arresting a spontaneous outbreak of pneumonia among stock monkeys was reported by Wisner,[1525] who injected subcutaneously three doses of a saline suspension of heat-killed cultures of the strain of Pneumococcus isolated from the infected laboratory animals.

appeared to render the individual more susceptible to infection. Lister,[816] however, found that 40 billion organisms could be injected into man without eliciting any but very slight reactions. In Ferguson's[436] experimental studies on man, 150 million organisms each of Types I, II, and III were injected subcutaneously at weekly intervals. Barach's[75] studies were carried out with doses of 200 million or more cocci at each injection. Duckwall[339] administered doses as small as 500 organisms at the first injection, increasing the amount to only 1,000 cocci on two subsequent occasions. In the study made by McCoy, Hasseltine, Wadsworth, and Kirkbride,[875] the lipovaccine used contained approximately 10 billion organisms of Types I, II, and III. Howell[661] standardized his preparation on the basis of dried pneumococcal protein and found that one cubic centimeter of vaccine containing 0.83 milligram of protein was a satisfactory dose. The circulars of directions accompanying the pneumococcal vaccines supplied by some of the commercial laboratories usually recommend the initial injection, in the case of a preparation containing pneumococci of the first three types only, of an amount representing 4,500 million organisms. For second and third doses the number is increased to 9,000 million pneumococci. In the case of vaccines consisting of pneumococci mixed with other bacteria of respiratory origin, the first dose represents 50 million pneumococci combined with 75 million for the second, third, and fourth injections. It appears, therefore, that pneumococcal vaccines may be safely administered within a wide range of dosage.

SPECIAL CONSTITUENTS OF PNEUMOCOCCUS

In the chapter on antigenicity, the immunizing action of certain isolated components of the pneumococcal cell was described. It may be remembered that Francis and Tillett[478] and Finland and Sutliff[448-9] succeeded in inducing the development of specific antibodies in man by the intradermal injection of soluble specific substance. Francis,[474] by injecting two groups of normal human be-

ings, one with the old form of capsular polysaccharide and the other with the new acetylated form, confirmed the observation that the deacetylated type of polysaccharide was antigenic for man, and ascertained that the new form possessed similar immunizing properties. More recently, Felton (1934)[417] tested the antigenic action of the acid-soluble portion of the pneumococcal cell on experimental animals and man, and reported that a small amount of the alcohol precipitate of the acid-soluble fraction immunized the test subjects to a degree approaching that possessed by patients convalescing from pneumonia. In a study with Sutliff and Steele, Felton[434] compared the immunizing effect of various fractions prepared from pneumococci with that of vaccines made from the intact cell. Although the optimal dose was not established, the injection of two milligrams of the soluble antigen produced as high a protective titer in the human subjects so treated as did the usual single dose of vaccine containing pneumococci. The authors also reported that under certain conditions the antibodies evoked by the soluble antigens prepared from pneumococci of Types I and II were heterologous as well as homologous in type.

POTENCY TESTS ON VACCINES

In order to obtain a measure of the immunizing power of pneumococcal vaccines, Bengston (1924)[99] studied the immune effects appearing in mice and rabbits after the administration of preparations obtained in the market. After testing the agglutinin, precipitin, and tropin content of the serum of the test animals as well as their ability to withstand subsequent infection with virulent pneumococci, Bengston concluded that the determination of active immunity in mice gave the best estimate of antigenic strength, particularly of polyvalent pneumococcal vaccines. The superiority of mice over rabbits could be ascribed to the fact that the virulence of the three fixed types can be maintained at a uniform level for the first-named species. In testing separately experimental vaccines prepared with organisms of Types I, II, and III, it was

found that the highest degree of protection followed in the case of Type I vaccine, less was afforded by suspensions of Type II cocci, while Type III preparations induced the least protection. In testing polyvalent vaccines, Bengston observed that their use resulted in more definite protection against pneumococci of Types II and III than was noted after the use of monovalent vaccines, suggesting the production of a certain amount of cross-protection. The tests, as a whole, showed that commercial polyvalent pneumococcal vaccines afforded protection in mice against amounts of virulent cultures ranging from zero to one million fatal doses in the case of Type I organisms, from zero to ten thousand fatal doses in the case of Type II, and only from zero to one hundred fatal doses of Type III cultures.

Before employing vaccines or other immunizing agents made from pneumococci, the preparations, as is the case with other biological products, should be subjected to some suitable test or tests to determine their antigenic strength. While it is obvious that effects in animals cannot be accurately translated into effects on man, yet determinations on laboratory animals furnish information concerning the probable immunizing action of bacterial agents or derivatives in the human body.

ROUTE AND SPACING OF INJECTIONS

Animal experimentation has demonstrated that the intravenous introduction of pneumococcal antigens, as compared to other methods, stimulates a more rapid and energetic immunizing response. The technique, despite certain and largely avoidable hazards, is applicable to man. As a matter of convenience, however, the subcutaneous route, especially in the prophylactic treatment of large numbers of individuals, is to be preferred. The intradermal injection of pneumococci, as shown experimentally in rabbits by Goodner,[525-6, 529] and of soluble specific substance, as demonstrated in man, by Francis,[474] Francis and Tillett,[478] and by Finland and Sutliff,[446-7] calls forth the prompt appearance of specific

antibodies. In this connection there may be also mentioned the intradermal injection in the treatment of lobar pneumonia of "natural bacterial antigens" prepared from living pneumococci by the addition of Merthiolate, as reported by Sutton, Kendall, and Rosenblum (1931).[1366] The results invite further study of the desirability of the skin as a locus for the administration of pneumococcal antigens.

That a certain degree of specific immunity in rats may follow the ingestion of pneumococcal derivatives has been shown by McDaniels,[876] among others, who, as a preparatory measure, fed egg-white to the animals thirty minutes previous to the oral administration of antigen. Animals receiving the preliminary dose of egg-white exhibited greater resistance than did the controls to a subsequent intraperitoneal injection of graded amounts of a virulent culture of pneumococci of homologous type.

In a series of communications extending over the period from 1925 to 1934, Ross[1178-94] reported the results of studies on the immunizing effect of feeding several different forms of pneumococcal material first to rats and subsequently to man. In some of the author's early experiments it was found that the ingestion of soluble specific substance from Type I pneumococci induced in the rat active immunity of homologous type, and a similar condition ensued after feeding the animals with filtrates obtained from pneumococci dissolved by bile salts. The degree of immunity following the administration of the polysaccharide appeared to be greater than that developing when suspensions of the intact cell were employed. In the case of Type II polysaccharide the animals acquired little or no immunity, but with Type III carbohydrate the effect, though slight, was type-specific. Ross noted that a large part of the polysaccharide administered orally passes through the alimentary tract practically unchanged as far as antigenicity is concerned but, strange to say, he could not detect the substance in the blood of the treated animals.

In his later publications, Ross reported the appearance of spe-

cific antibodies in the serum of man following the administration of pneumococcal vaccines by mouth. The preparations employed comprised suspensions of pneumococci freshly killed by treatment with hydrochloric acid; suspensions of the same organisms after desiccation; bile solutions, sometimes filtered, of the cocci; and dried and pulverized preparations of these solutions. In addition, in some experiments, Ross fed to the human subjects living pneumococci either dried and contained in capsules or suspended in water. The results of the later experiments on human beings reported by Ross may be epitomized thus: Vaccines administered by mouth in the case of Type I materials induced antibody formation in 74 per cent of fifty-three persons, seven of whom required two series of feeding, while in fourteen other persons no protective antibodies appeared after two series comprising ten feedings. Sixty per cent of individuals receiving Type II antigens developed antibody, while eleven of the subjects failed to react, although five were given two series by ingestion. With Type III, the results were difficult to evaluate since antibody production was slight and irregular. The content of antibody in the blood of men orally immunized was low and was similar in degree to that of convalescent blood, while variations in the amount of antibody were observed in different individuals. Ross noted heterogeneity of the antibodies appearing as a result of vaccines administered orally, especially with those derived from Type II organisms. The duration of the immunity developed after the ingestion of the various preparations was approximately seven to fourteen months, but the experimental evidence bearing on this phase of the subject is not complete. Ross recommended as a practical procedure the use of completely autolyzed cultures, because he believed that autolysates contain several times as much antigenic material as eighteen-hour cultures of cocci in broth. From all that we know of the antigenic integrity of the components of Pneumococcus, it is difficult to subscribe to this recommendation.

During the period in which Ross was prosecuting his studies,

Kolmer and Rule[746-7] were also investigating the comparative efficacy as immunizing agents of suspensions of killed pneumococci of Types I, II, and III. Vaccines containing cultures devitalized by tricresol were injected subcutaneously at intervals of five days into one series of rabbits and similar injections of organisms treated with hydrochloric acid were given through a stomach tube to animals of another series. Although the two immunizing agents were not strictly comparable, the observations led the authors to conclude that the antigenic action of the vaccines was more pronounced when administered by the subcutaneous than by the oral route. The best results were obtained with a minimum of five daily injections. The work of Ross and that of Kolmer and Rule present no evidence of the advantage, save possibly that of the ease of administration, of ingestion over injection of pneumococcal vaccines.

While intratracheal insufflation, as practiced by Cecil and Steffen,[214-5] is of interest in demonstrating the possibility of inducing immunity by local stimulation, the method obviously is scarcely applicable in the case of man.

LOCAL AND SYSTEMIC REACTIONS

Pneumococcal substance, unless in autolyzed or degraded form, is not conspicuously toxic when introduced parenterally into the tissues of man, and vaccines made from pneumococci or some of their constituents (unlike similar preparations prepared from such bacteria as the dysentery and paratyphoid bacilli, the bacillus of Pfeiffer, and *Bacillus proteus*) can be administered in the customary amounts without risk of incurring undesirable or unduly severe reactions. Cecil[201, 203] spoke of both local and general toxic manifestations following the subcutaneous injection of saline suspensions of pneumococci of the first three types when the dosage equalled several billion organisms. The subjects exhibited variations in reactivity, and the severity of the effect could be lessened by decreasing the individual dose. Malone[864] reported that

less than 2 per cent of Senegalese troops reported sick after the injection of 3,000 million to 6,000 million cocci.

In the study by Von Sholly and Park,[1452] 63 per cent of the subjects vaccinated with a mixed vaccine developed after the first dose mild reactions consisting of redness and soreness of the arm; the same number had similar reactions following the second and larger dose; while the third injection elicited a comparable condition in 60 per cent of the subjects. Constitutional reactions as evidenced by headache, malaise, vertigo, chills, and general pain occurred in 19 per cent of the treated subjects after the first injection, in 27 per cent after the second, and in 23 per cent after the third dose. In a later tabulation, Park[1054-6] listed severe reactions as appearing in 3 per cent of the individuals after the first injection, 2.7 per cent following the second, and 4.1 per cent after the third dose. It should be borne in mind that the vaccine contained, in addition to pneumococci of the first three types, streptococci of the hemolytic and viridans type and the so-called influenza bacillus. One cannot say to what extent these organisms participated in the toxic action of the vaccine. Lister and Ordman,[819] also using a mixed vaccine, stated that in their experience while mild reactions were frequently noted severe reactions were uncommon. Barach (1931)[76] encountered no immediate reactions after the intravenous or intradermal administration of suspensions or filtrates of heat-killed pneumococci. A chill was later observed in one patient so treated. Although other references might be quoted, there appear no reports of reactions following the injection of pneumococcal vaccines sufficiently severe or untoward as to preclude their use.

APPEARANCE AND DURATION OF VACCINAL IMMUNITY

One of the noteworthy phenomena of artificially induced, active immunity to Pneumococcus is the early appearance of specific antibodies in the circulating blood following the parenteral injection of pneumococcal antigens. Barach (1928)[74-5] was able to detect type-specific protective substances in the serum of rabbits

three days after the injection of a vaccine consisting of sterilized, intact organisms of Types I and II, while similar immune effects were demonstrable on the fourth day after the administration of filtrates of broth cultures of the cocci. In an experimental way, Goodner[525-6, 529] showed that protective and other antibodies were present in the blood of rabbits in one to two days after intradermal inoculation with living pneumococci. In immunization tests carried out on man with various antigenic preparations made from Type I and Type II pneumococci, Felton, Sutliff, and Steele[434] found that antibodies appeared in the blood stream to a slight extent on the fourth or fifth day after injection and increased up to a maximum on the fourteenth day. The literature contains many references to the early development of humoral, immune substances or of increased resistance engendered by the administration of pneumococcal antigens, but it seems scarcely necessary to relate them.

The statement was made earlier in this chapter that the duration of the immune state established by the administration of pneumococcal vaccines, as far as present evidence indicated, could not be expected to surpass that arising during an attack of pneumonia. All the experiments in which this time element was noted bear witness to the validity of the statement. Maynard,[872] basing his observations on the incidence of natural immunity in vaccinated natives in the Rand as compared to that of unvaccinated natives, reported that the protection conferred by the immunization treatment appeared to be greatest immediately or shortly after vaccination and then progressively diminished, until after a period of about four months it was lost. In the rabbit, following the intradermal injection of living pneumococci, Goodner could no longer demonstrate the existence of active immunity after a lapse of fifty to sixty days. Although the presence of demonstrable specific antibodies in the serum of a vaccinated individual may or may not be an index of resistance to pneumococci, their appearance and persistence bear witness to the fact that the physiological

mechanism of the vaccinated person has been operating in the production of immunity.

On this basis, Howell[661] determined the agglutinative, complement-fixing, and protective titer of the serum of three healthy men who had received by subcutaneous injection one cubic centimeter of the American Army lipovaccine, containing 0.83 milligram of dried pneumococcal protein of Types I, II, and III. There was a definite rise in agglutinins in the second week after injection, the height being reached from the second to fourth week, although the antibody was still demonstrable from three to nine months. The complement-fixation reaction with the subjects' serum became positive from the seventh to the fifteenth day, was strongest at the twenty-fifth day, and still was positive over the period during which agglutinins were shown to endure. The content of protective antibodies in the serum more or less paralleled that of agglutinins.

When heat-killed vaccines were administered intravenously in rabbits and the animals subsequently tested by intradermal inoculation, Stillman and Goodner[1342] showed that the immune effects persisted for a somewhat longer time. In the case of Type I vaccines, the resistance induced appeared to be high ten days after the completion of the course of injections, then to become irregular in degree and generally lower during the next four months. After the seventh month, the level of resistance dropped again and was usually low. The content of protective antibodies paralleled the degree of resistance of the animals throughout the period of observation but the agglutinin titer of the treated animals' serum was high only during the first two months. With Type II vaccines, the resistance to infection was somewhat lower than that evoked by Type I vaccine, but remained moderately high for nine months with an abrupt decrease after that period. Treatment with Type III preparations resulted in a fairly marked degree of resistance during the first month, which, however, diminished sharply and remained low. None of the animals treated with Type III vaccine exhibited agglutinins or protective antibodies in the serum. While

there appeared to be some correlation between the amount of humoral antibodies and the degree of resistance, the exception in the case of animals treated with Type III agents, and individual differences noted in animals as far as this parallelism was concerned, led the authors to conclude that, in addition to differences in the antigenic capacity of strains of various serological types, one must also consider the operation of cellular as well as of humoral immunity in resistance to infection.

In the experiments of Ross already cited, antibodies appearing in the serum of human beings after the ingestion of pneumococcal vaccines disappeared in some individuals within six months, and in others endured for approximately two years. Felton, Sutliff, and Steele[434] found that the presence of immune substances in the blood of human subjects, injected subcutaneously with the several preparations selected, was appreciable when the serum was tested three months after the immunizing treatment. However, the observations just quoted do not necessarily denote that resistance to infection persisted for so long a time.

There would be no object in multiplying the number of references bearing on the duration of active immunity conferred on man by the administration of vaccinal preparations made from pneumococci. Sufficient has been said to indicate that resistance, while increasing soon after injection, as far as the presence of circulating protective substances enters into the reckoning, may be naturally destroyed within the body or, at least, fail to be demonstrable. The period of active immunity is short as compared to the immunity induced by the injection of toxins or of some filtrable viruses.

RESULTS FOLLOWING VACCINATION IN MAN

In discussing the biology of Pneumococcus, it is not the intention of the authors of the present volume to invade the fields of preventive or clinical medicine. Notwithstanding this reservation, in order that the allied subjects of antigenicity and of antibody

formation may be followed to their logical end, and also for the information of readers who may not have access to original source material, the results attending the use of vaccines for the prevention and cure of pneumococcal disease will be dealt with in summarized form.

PROPHYLAXIS

Because of the enormous toll exacted by pneumonia among the ranks of native workers in the diamond mines in South Africa, Maynard (1913)[872] sought whatever benefit might accrue to the men by the injection of vaccines prepared from Pneumococcus. In the beginning of the investigation, two doses of 40 to 60 million, then of 100 to 200 million, and finally of 300 million organisms given immediately upon the arrival of the natives on the Rand appeared to reduce the incidence of pneumonia for a period lasting not over four months. There was no evidence that the preventive treatment influenced case fatality, except possibly for a very short time after injection. On account of historical interest and because of the contributions of the senior author to the subject of bacterial vaccines, the work of Wright, Morgan, Colebrook, and Dodgson (1914)[1545-6] should be cited. Administering heat-killed broth cultures in subcutaneous doses representing 300 million to 600 million pneumococci to several thousand native Africans in the Rand mine, the authors reported an apparent decrease of approximately 50 to 60 per cent in the incidence of pneumonia among the vaccinated men when compared to the attack-rate among untreated controls.

During the mobilization of American troops at Camp Upton during the World War, Cecil and Austin[204] vaccinated more than twelve thousand men, giving three or four injections at intervals of five to seven days of suspensions of heat-killed cultures of Type I, II, and III pneumococci. The total dosage consisted of six to nine billion organisms of Types I and II and four and one-half to six billion of Type III pneumococci. In the ten weeks which elapsed

after vaccination, no cases of pneumonia of the three specific types developed among the men who had received two or more injections, whereas there occurred twenty-six cases due to pneumococci of the same types among the nineteen thousand or more unvaccinated soldiers. The case mortality of all types of pneumococcal pneumonia among the vaccinated was 11.7 per cent against a mortality of 28.0 per cent among the unvaccinated troops.

The results following the use by Cecil and Vaughan[216] at Camp Wheeler of lipovaccine in a single dose containing approximately ten billion each of pneumococci of Types I, II, and III were not so striking as those at Camp Upton. Men representing 80 per cent of the camp strength—a total of 13,460—received the vaccine treatment and among them, during the two or three months following the injection, thirty-two cases of pneumonia caused by the first three types of Pneumococcus developed, whereas among the unvaccinated soldiers, comprising one-fifth of the whole group, there were forty-two cases in the same period. The death-rate from all pneumonias due to different types of pneumococci occurring among the vaccinated men one week or more after the single injection was 12.2 per cent, whereas among the unvaccinated control subjects the percentage was 22.3. Of the primary pneumonias among the vaccinated troops, the fatality-rate was 11.9 per cent as compared to 31.8 per cent in the case of the untreated men. In their deductions from the records, Cecil and Vaughan suggested as possible causes for the less favorable results the fact that at Camp Wheeler many of the soldiers were raw recruits, a large part of whom were Negroes of rural origin, whereas at Camp Upton the men were largely white recruits from New York City and were well seasoned at the time of vaccination. The authors made no reference to the part which the use of lipovaccine instead of saline bacterial suspension or the single injection as against two or more injections may have contributed to the less favorable experience.

In regard to the use of lipovaccines, the report of Rosenow and Sturdivant (1919)[1173] may be quoted. Three subcutaneous injec-

tions were given at weekly intervals of a vaccine in an oil men-
struum containing 30 per cent of pneumococci of Types I, II, and
III, 40 per cent of Group IV, 20 per cent of hemolytic strepto-
cocci, and 10 per cent of *Staphylococcus aureus*, representing in-
dividual doses of 2.5, 5.0, and 7.5 billion cocci respectively. The
authors admitted that the collected data on the 93,476 individuals
vaccinated were far from exact; nevertheless, the figures would
seem to be significant. The average incidence of influenza and pneu-
monia in the group receiving three injections was one-third that of
the incidence among the unvaccinated subjects, and the average
mortality-rate for the treated was about one-fifth that for the un-
treated group.

Another Army experience was that at Camp Taylor (U.S.A.)
reported in 1920 by Duckwall,[339] who employed as vaccine a saline
suspension containing in each cubic centimeter one thousand or-
ganisms of heat-killed pneumococci of Types I, II, and III. For
the 1,326 men receiving one, two, or three injections of vaccine at
intervals of seven to ten days the annual admission-rate per thou-
sand from pneumonia was 20.3, and for those receiving no prophy-
lactic treatment the rate was 54. The death-rate, similarly calcu-
lated, was 2.26 for the vaccinated and 8.76 for the unvaccinated
subjects. Considering the statistics for pneumonia together with
those for common respiratory infections, the author stated, "The
obvious conclusion is that the vaccine protects against pneumonia
and the common respiratory diseases in the proportion of five to
one." In comparison with the dosage employed by other observers,
that used by Duckwall is conspicuously small.

In the same year, Borrell[143] described the effect of the adminis-
tration of saline suspensions of pneumococci (types not given) to
Senegalese troops stationed in France during the World War.
When two injections of a vaccine representing 3,000 and 6,000
million cocci were injected at seven-day intervals, the morbidity-
rate per thousand was thirty-six and seventy-three respectively
among the vaccinated in two battalions as against one hundred

thirty and eighty-five among the troops not vaccinated. The mor-
tality-rate on the same basis was zero and seven as compared to
thirty-three and eighteen. Later, when the number of injections
was increased to three, consisting of doses of 0.5, 1.0, and 2.0
cubic centimeters of a suspension containing 8,000 million organ-
isms per cubic centimeter, the results were not so satisfactory; the
morbidity-rate was sixty-five among the vaccinated as compared
to a rate of ninety-five among the unvaccinated soldiers and the
mortality-rate was nineteen among the treated subjects and
ninety-five for the controls.

In 1921, Von Sholly and Park[1452] reported their experience in
vaccinating 1,536 employees of the Metropolitan Life Insurance
Company in New York City. Of the employees, 1,412 received
three injections of a saline suspension of pneumococci of the first
three types sterilized by heat, and the other 124 were given lipo-
vaccine prepared by Rosenow. Among 1,327 complete records
there was found one case of pneumonia among those vaccinated
against eleven cases among the control groups. The treatment ap-
peared to have little effect either in preventing or in favoring re-
spiratory infection other than pneumonia among the subjects
treated.

In its chronological order there may be mentioned the note of
Walravens,[1477] which stated that at Katanga in the Belgian Congo
it was the routine practice in large industrial concerns to give the
black employees prophylactic injections of vaccines made with
cultures obtained from Lister and with other strains of local iso-
lation.

In 1922, Field[439] communicated the results following the use of a
mixed vaccine among the troops stationed at Fort Myer, Virginia.
The preparation contained pneumococci of Types I and II, he-
molytic streptococci, and the Pfeiffer bacillus, to a total of 6,000
million organisms per cubic centimeter, and was administered in a
first dose of 0.5 cubic centimeter followed by two subsequent in-
jections of one cubic centimeter each. The statistics presented by

Field are not highly conclusive, but it was his impression that a positive effect in lowering the incidence of pneumonia was obtained by means of the vaccine.

Of more significance is the report of the study made by Mc-Coy, Hasseltine, Wadsworth, and Kirkbride.[875] To 17,752 inmates of various state institutions was given a single subcutaneous injection of one cubic centimeter of lipovaccine containing approximately ten billion pneumococci of the first three types, while 18,595 subjects and 7,992 newly admitted patients were reserved, untreated, as controls. Observations extended over a period of two years, and in the final analysis of the data it appeared that of the cases of pneumonia occurring among the vaccinated subjects 18.0 per cent were caused by organisms of Types I, II, and III, while in the control series the percentage was 23.6—scarcely a satisfactory degree of protection. From theoretical considerations the comparative failure of lipovaccines may be ascribed to lower antigenic potency of bacteria when suspended in oil—an opinion shared by many immunologists and expressed by Cecil in 1923.*

In a paper by Malone[864] and in one by King[714] appearing in 1925, the view was expressed, based on the results of the vaccination of 2,500 troops in India, that vaccination as practiced was probably without any protective value. The vaccine contained only pneumococci of Types I and II in such a concentration that the dose, given twice subcutaneously at an interval of a week, contained 5,000 million cocci. A case-rate among the vaccinated of 9.3 when compared to the only very slightly higher rate of 10.7 for the unvaccinated controls would justify the authors' unfavorable opinion. However, a less pessimistic view concerning the value of vaccines in the prevention of pneumococcal infections was expressed by Cole in 1920,[262] who also in 1934[266] concluded: "It is not impossible that, even with our present knowledge, something could be accomplished by vaccination." Cole, in discussing the

* A review of the prophylactic vaccine treatment of pneumonia was published by Cecil[203] in *Medicine* in 1925.

soluble specific substance of Pneumococcus, stated that possibly
the polysaccharide might prove to be useful for preventive treat-
ment of man.

Another aspect of the possible effect accomplished by the large-
scale prophylactic treatment of man with pneumococcal vaccines
was suggested by Lister[818] in 1929, who observed that since the in-
fluenza pandemic of 1918 the types of Pneumococcus, previously
found by him to be responsible for more than 69 per cent of the
cases of lobar pneumonia in the Rand, had practically disap-
peared, and he attributed the change in the pneumococcal flora of
the native workers to the routine practice of vaccination carried
on since 1918. The disappearance of the familiar types of pneu-
mococci against which vaccine treatment had been directed and the
appearance of miscellaneous types in the cases of lobar pneu-
monia then occurring was commented upon by Ordman[1033] in 1931.
The morbidity and mortality rates from the disease decreased and
there was a modification of the character of the pulmonary infec-
tion. Ordman, like Lister, believed that these changes had been
brought about by the persistent vaccination of the natives. It then
became the custom in the diamond mines to treat the native work-
ers entering the mines in summer and autumn with the usual vac-
cines and to inject those entering in winter and spring with mixed
bacterial vaccines, designated "community autogenous vaccines,"
containing streptococci and the influenza bacillus.

In a review of the various attempts to protect the miners in the
Rand from pneumonia, Orenstein[1035] in 1931 expressed frank skep-
ticism concerning the value of the procedure. He had doubts about
the importance of the serological classification of pneumococci, he
saw a serious complication presented by the multiplicity of types,
and was somewhat pessimistic about the alleged immunizing action
of pneumococcal antigens as shown by animal experiments. Never-
theless, in the next year, Orenstein[1036] reported on the use of mixed
vaccines begun experimentally the year before, and from statistics
on 40,000 natives so treated suggested that those receiving the

mixed vaccine showed decreased pneumonia mortality. A fair criticism of the work of both Ordman and Orenstein would be the incompleteness of their studies of the natural bacterial flora of the natives both in the population at large and among the workers before and after prophylactic vaccination. Furthermore, as is the case in practically all studies of mass vaccination, the communications include no observations on the production of specific antibodies in the vaccinated subjects.*

Less discouraging was the experience of Peall (1935).[1075] During a three and one-half year period, over 68,000 native workers in the Randfontein mines were given, at weekly intervals, three doses of a mixed vaccine containing pneumococci—the "community autogenous vaccine"—and there occurred a prompt and marked drop amounting to 70 to 80 per cent in the incidence and fatality-rates of pneumonia and of other respiratory infections as well. Despite previous doubts, Ordman[1034] resumed his endeavors toward the prevention of pneumonia and other respiratory disease, and this time among a presumably highly susceptible class of natives—workers who had been prohibited by the South African government from working in the Rand gold fields because of the extremely high pneumonia rates prevailing among them. Previous to prophylactic vaccination, the annual attack-rate was from fifty to eighty per thousand and the mortality-rate eighteen to twenty per thousand. After the continued use of a mixed vaccine containing only one type (Type II or Group B) of Pneumococcus combined with two varieties of streptococci, *Micrococcus catarrhalis* and *Staphylococcus aureus*, administered in three weekly doses, the fatality-rate from pneumonia fell from 13.9 to 2.4 per thousand—a reduction of 82.7 per cent.

A recent historical review, summarizing the preventive work done in the Rand and neighboring mines and reporting the latest experience of the authors, was published in 1935 by Lister and

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* For a general review of the many phases of the use of vaccines in warding off pneumococcal infections or in their cure, the reader is referred to Park's third Harben Lecture.[1056]

Ordman.[319] Bacteriological studies had shown the presence among the native mine population of eighteen types or groups of pneumococci other than the Lister Groups C, B, and E (the classical Types I, II, and III). The vaccine, therefore, was made to include eight groups of pneumococci and, in addition, strains of streptococci, the Pfeiffer bacillus, *M. catarrhalis*, *Staphylococcus aureus*, and the Friedländer bacillus. The cultures were suspended in salt solution, sterilized by one hour's heating at 60°, and preserved with phenol. The vaccine was injected subcutaneously in three doses at seven-day intervals. The results up to the date of the report were sufficiently encouraging to lead Lister and Ordman to believe that the practice would make possible the employment of tropical natives formerly barred from the mines because of their unusual susceptibility to respiratory infections.

Early in 1935, Felton[418] made a preliminary announcement to the effect that he had tested various water-soluble fractions of Pneumococcus for their immunizing action on man, but the nature of the preparations was not revealed. A single subcutaneous injection of two milligrams of the preparation was given, and in a group of two hundred persons antibody was found to be present in the serum of all individuals so treated fourteen days after injection. The antibody was still demonstrable in the majority of subjects after a lapse of three months and in a few tested after ten months. The amount of antibody found as compared to that originally present in the serum of the subjects varied from a seventeen-fold to a ninety-eight-fold increase. All the preparations employed were high in polysaccharide content, but Felton, at the time, had not decided whether the antigenicity of the immunizing agents was due to the polysaccharide, to the polysaccharide combined with some other substance, or to some other cell constituent or product.

In a more complete report published in the same year, Felton with Sutliff and Steele[434] presented the details of preparation of a variety of antigens derived from pneumococci of Types I and II. A single subcutaneous injection of the different agents was given

to human beings and the antibody response measured by testing the protective strength of the serum of the subjects so treated. The results of the investigation proved that immunity against pneumococci may be produced in man by the injection of soluble products prepared from the microorganisms. Among the pneumococcal materials employed was a water-soluble substance that failed to give biuret and Molisch reactions,* lacked the property of precipitating immune serum, and yet immunized white mice. The substance was considered by the authors to differ from any hitherto studied fraction of Pneumococcus. All the preparations tested for antigenicity, as well as dried organisms, evoked an increase in protective antibodies in the serum of the individuals treated. The degree of immunity established from a single injection of a two-milligram dose of antigenic substance, as measured by protective antibodies in the serum, compared favorably with that found in the serum of convalescent pneumonia patients. The duration of the immunity following the use of vaccine or of the fractions employed varied in different individuals, but in some cases persisted for at least a period of three months.

Wadsworth,[1465] in a discussion of the practical limitations of the vaccinal prevention of pneumococcal infection, differing with the opinion of Lister, Ordman, and others, stated that with rare exceptions pneumonia does not yield to any form of prevention and that the protective inoculation with pneumococcal vaccine advocated by some authorities as a public health measure is not practical. Granting the soundness of the latter portion of the statement, prevention, nevertheless, as a measure for sparing the individual from an attack of pneumonia is not to be dismissed so lightly. The very definite limitations of this method of prophylaxis, such as the heterogeneity of serological types and the brief spell of immunity following vaccination are freely admitted. However, in the case of individuals debilitated by age, by the effects of

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* In another part of the same paper the authors stated that the substance might contain a polysaccharide as a part of its constituency.

disturbances of nutritional function, by intercurrent or chronic disease, or by undue physical strain, to whom pneumonia may present an especially serious hazard, and in the case of large bodies of men assembled from many sections of the country and living for a time under crowded conditions, the protection, such as it is, afforded by the parenteral administration of suitable pneumococcal materials should not be disregarded. To be sure, the biological agent should be prepared with proper attention to the type or types of Pneumococcus prevalent at the time, the kind of vaccine selected should represent the best immunological practice, and the injections should be repeated at frequent intervals. The danger of pneumonia to man both individually and in masses merits the application of such preventive measures as we possess, imperfect as they are, until an antigen more closely approaching the ideal is devised.

VACCINE TREATMENT OF PNEUMONIA

The possible curative action of bacterial vaccines in pneumococcal infections more properly belongs to a discussion of the clinical features of the disease. The reader in search of this information is referred to the volume by Heffron,[601] where a more exhaustive treatment of the subject will be found. However, in order to round out the description of the many biological activities of Pneumococcus, there are included in the present chapter references to the effect of specific vaccines administered during the course of pneumococcal disease, but only a few of the many observations will be noted. The rationale of vaccine therapy in pneumonia is based on the unusually rapid development of type-specific antibodies created by the parenteral injection of suitable antigenic agents. The introduction of pneumococcal antigens into a body already infected with millions of pneumococci and therefore having its tissues permeated by the antigenic constituents of the bacteria in their native condition would, because of certain theoretical considerations, seem paradoxical. There is the possibility,

however, that these stimulating substances in modified form, when introduced into the cutaneous or subcutaneous tissues or into the blood stream before circulatory invasion by the cocci has taken place, may aid in fortifying the natural defenses of the body by stimulating the rapid development of substances antagonistic to the invading bacteria.

In the clinical and statistical report published by Orenstein[1035] in 1931, the author stated that the experimental treatment of pneumonia patients with pneumococcal vaccine failed to influence favorably the mortality from the disease. Lister,[816] on the contrary, in 1916 reported that the intravenous injection of saline suspensions of killed pneumococci into seven patients suffering from lobar pneumonia caused no negative phase and apparently exerted a beneficial effect, since all the patients recovered.

Rosenow (1918),[1171] reasoning from his animal experiments that partial autolysates of virulent strains of the different types of pneumococci might better serve as auxiliaries in the mobilization of protective substances during an attack of lobar pneumonia, applied this form of treatment to pneumonia patients studied during three consecutive winters. The antigen was prepared by suspending in salt solution the sediment from cocci cultivated for eighteen to twenty-four hours in glucose broth, then allowing the suspension to autolyze at incubator temperature under a layer of ether. Self-digestion was permitted to progress until 95 per cent of the organisms became Gram-negative and five cubic centimeters of the preparation produced few or no symptoms in medium-sized guinea pigs. The dosage, in practice, was one cubic centimeter daily until the temperature became normal. The results, according to Rosenow, were surprisingly good: of ninety-five patients receiving the first injection of vaccine within forty-eight hours after onset of the disease only 3 per cent died; of one hundred five injected on the third day of the attack or later the mortality was 11 per cent. Furthermore, the administration of vaccine shortened the duration of the disease depending upon the promptness with which the treat-

ment was begun. In the paper there was no mention of type determinations of the infecting pneumococci.

In a detailed clinical report on the use of a mixed vaccine in over two hundred pneumonia patients studied during the period 1922 to 1926, Lambert[776] published the following fatality-rates: In cases treated within the first forty-eight hours of the disease the figure was 5.8 per cent; for those patients receiving the vaccine in the first seventy-two hours the percentage was 9.8; when treatment was first instituted when the disease had been present for seventy-two hours or more 26.2 per cent of the patients died, giving a fatality-rate of 21.2 per cent for the entire series of cases treated with autolysate as against a rate of approximately 40 per cent for the untreated controls.

The significance of the results is somewhat obscured by the fact that the vaccine which Lambert employed, in addition to containing forty strains of pneumococci of the first three types and Group IV, also included four times as many other organisms such as streptococci, staphylococci, *Micrococcus catarrhalis*, and Pfeiffer bacilli. Whatever specific effect there was could be attributed to the pneumococci in the vaccine, while such influence as the bacteria of heterologous species may have had on secondary invaders might conceivably have contributed to the results. Lambert's recommendation that stock, mixed vaccines be used removes this form of therapy from the domain of scientific treatment and places it in the realm of empiricism. In Park's Harben Lecture,[1056] already quoted, he stated that a vaccine composed of several types of pneumococci and of other bacteria of respiratory origin in the treatment of lobar pneumonia was without appreciable effect.

Barach's[76] series of cases was small but his report may be included as lending some, though slight, support to the claims of advocates of vaccine treatment in pneumonia. Barach gave to twenty patients intravenous injections of monovalent vaccines representing both heat-killed cultures of pneumococci of Types I, II, and III and filtrates prepared from suspensions of the same organisms.

Two deaths occurred in the series; in one case, a vaccine of homologous type had been administered and, in the other, an heterologous preparation. Sutton and associates[1366] claimed favorable results attending the use of a "natural" pneumococcal antigen. The mortality among the treated cases was 22.5 per cent whereas 42 per cent of the untreated controls died.

Before any final appraisal can be made of the value of pneumococcal vaccines in the treatment of lobar pneumonia there must be more evidence at hand, and the evidence must be supplied by carefully controlled clinical studies in which large series of cases of which some groups are given monovalent preparations of pneumococci of a type homologous with that infecting the patient; other groups should receive vaccines of pneumococci of a variety of serological types without admixture of other bacteria; others should be given similar vaccines combined with organisms of respiratory origin; while the results in all the cases in the groups studied should be compared with the fate of a group of pneumonia patients who receive no pneumococcal antigens. Furthermore, type-determinations and blood cultures should be carried out in all cases studied. Without accurate and thorough observation of all the manifestations following the administration of the specific agent with proper bacteriological and serological controls no sound verdict can be reached.

CHEMOTHERAPY

The action in vitro *and* in vivo *of various organic and inorganic compounds on Pneumococcus, and their use in the treatment of pneumococcal infections.*

IT would be a digression—a digression far beyond the scope of this book—to treat at any length the administration of drugs for the cure of infections due to pneumococci. It is assumed that the reader has a special interest in the effects of chemical agents on the life processes of Pneumococcus; for those whose interest extends to the more strictly therapeutic features of pneumococcal disease, Heffron's *Pneumonia*[601] will be found to contain an exhaustive discussion of the subject.

In the chemical treatment of diseases of parasitic origin, the agents usually employed fall into several main groups: arsenical preparations, modifications of alkaloids, derivatives from coal tar, metallic salts, and the usual pharmacopeial formulas. Those compounds have been chosen that exert a selective action on the invading parasite rather than those that stimulate normal physiological functions of the host; in other words, those that are specific rather than supportive. As with many other pathogenic bacteria, the great majority of chemical agents which are noxious to the pneumococcal cell are similarly injurious to the cells of animal tissues; many are too poisonous in the animal body, possess too narrow a margin of safety between therapeutic and toxic doses, or manifest such undesirable side or secondary actions that their use is precluded.

In pneumococcal infections the substances which have been studied for specific curative properties include bile and its component salts, some of the coal-tar dyes (especially the flavines), such metals as gold, silver, and iodine, and derivatives of the al-

kaloid, cinchona. Because of the relative unimportance of all chemical agents so far tested, with the exception of the cinchona compounds, the discussion of all but the last-named group will be presented in summarized form.

Chemical Agents Other Than Cinchona Derivatives

BILE

Powerful as is the action of whole bile or of sodium salts of the bile acids in the dissolution of the pneumococcal cell in the test tube, the lytic effect on pneumococci in the body is much less pronounced. Pneumococci, circulating in the blood or present in lesions or cavities accessible to the agent, may undergo lysis, but that is only one feature of the effect, because the physiological action of injected bile salts in a large measure offsets any benefits to be derived from the dissolution of the cocci. Barjot (1928)[79] treated a few cases of lobar pneumonia by the intravenous administration of sodium taurocholate in an artificial serum containing magnesium sulfate, but the small number of patients and the nature of the results would scarcely seem to justify the author's faith in the benefits to be derived from the treatment. Ziegler,[1570] employing saline solutions of the sodium salts of taurocholic and glycocholic acids, reported, as might be expected, a cholagogic effect along with erratic fluctuations in the temperature curves of the patients so treated. The bile salt caused damage to the vein at the point of injection, sometimes resulting in obliteration of the vessel, but its use was attended by no other appreciable toxic symptoms, although, to be sure, a slight degree of anemia was observed. In a second communication, Ziegler[1571] stated that in rabbits the intravenous injection of sodium dehydrocholate in the concentration employed was without the injurious action of the corresponding taurocholate and glycocholate on the tissues in the neighborhood of the injection, but apparently the treatment was not applied to human beings. Because of both the predictable local

and systemic effects of biliary salts these substances hold little promise as medicinal agents for the treatment of pneumococcal infections.

SOAP

So, too, with soaps. According to Lamar (1912),[775] sodium oleate when added to specific immune serum and boric acid, may exhibit an adjuvant action in combating pneumococcal infection of the meninges. The combination regularly exerted a more powerful action than immune serum alone and not only prevented the occurrence of infection, but also, when administered repeatedly, arrested the progress of an already established infection and often led to the recovery of the infected animals. The treatment, however, was not applied to meningitis in man. Lauric acid was shown by Walker[1476] to be strongly germicidal for pneumococci, while Larson and Nelson[791] in their study of sodium ricinoleate found that the soap was fatal to virulent cultures of the organism. These compounds, however, were not administered to human beings.

COAL-TAR DYES

The affinities possessed by bacterial cells for dyes render the cells susceptible to the bacteriostatic action of the dye retained by the cell after treatment by the Gram technique, but the action of the dye, even when mordanted, is rarely of such a nature as to affect the organism when circulating in the blood stream. Of the many substances studied with a view to ascertaining the pneumococcidal action in the circulation, dyes of the triphenylmethane series were found by Simon and Wood,[1288] in cultivation experiments, to be strongly inhibitory to the growth of pneumococci. Because of their antiseptic or germicidal properties, the flavines have received special attention from students of Pneumococcus. Schiemann and Baumgarten[1227] reported that acriflavine exerted a powerful, selective action on both virulent and avirulent members of

the species. Neufeld and Schiemann[999] showed that trypaflavine and different salts of 3–6 diamino-acridine were strongly bacteriostatic *in vitro. In vivo*, the compounds when administered subcutaneously rescued a certain percentage of mice infected intraperitoneally with pneumococci. The dyes were also efficacious when applied in the form of irrigations in wounds experimentally infected with pneumococci in mice and guinea pigs (Neufeld and Reinhardt,[996] and Reinhardt[1134]). From animal experiments, Schiemann,[1224] by intraperitoneal or topical application, substantiated the observation on the favorable influence exerted by trypaflavine on pneumococcal infection. Maeji[851-2] stated that trypaflavine was pneumococcidal in a dilution of 1 to 100,000. To the list of pneumococcostatic agents Schiemann added the aurophosphine and trimethane dyes, crystal violet and brilliant green. As for Mercurochrome, the few references (Freeman and Hoppe,[481] and Phelps[1088]), lacking any laboratory tests, are not significant, whereas the somewhat analogous substance, Metaphen, was reported by Kolmer and Borrow[742] to be less effective than optochin, a substance which will be discussed later. From the few references in the literature it would appear that such tinctorial substances derived from the nitrobenzines as have been studied manifest no conspicuous pneumococcidal properties when introduced into the animal body.

METALS AND METALLIC SALTS

Of metallic elements, gold in colloidal form has been tried in the treatment of pneumococcal infection in man (Gautier[505]), and as a synergist with specific serum in the treatment of experimental infection in rabbits (Gelarie and Sabin[510]). In the latter case, the survival ratio of the animals treated with serum and gold was greater than that of those receiving subeffective doses of serum alone. Schiemann and Feldt[1229] reported that gold in the form of Sanocrysin had slight if any action in protecting mice infected with pneumococci. Gautier mentioned iodine as a therapeutic agent in the treatment of pneumonia but offered little information con-

cerning the effect in combating pneumococcal infections. Iodine in colloidal solution was administered by Murphy[940] to ninety patients ill with pneumonia; of the ninety, ten died. The report contained no account of any *in vitro* experiments with the solution.

OTHER MEDICINAL AGENTS

Many drugs have been subjected to clinical trial, not on the basis of laboratory experiment, but apparently in the hope that some selective action on Pneumococcus might follow their administration. Reports on epinephrine have come from Puscariu and Nitzulescu,[1113] and on camphor by Rosenthal and Stein[1174] and by Welch and Rueck.* According to Boehncke,[133] the subcutaneous injection of camphor dissolved in oil was able to protect mice and rabbits against intraperitoneal and intravenous inoculation with virulent pneumococci made some hours later. Formaldehyde sulfoxylate which, according to Rosenthal,[1177] was without pneumococcidal action *in vitro*, nevertheless, when injected subcutaneously into mice at the time of inoculation in three or four-hour doses repeated daily, protected the animals from an otherwise fatal infection. Rosenthal,[1176] and Powell and Jamieson[1104] have also published the results of experiments with the same drug. Schottstaedt[1249] and physicians of the United Fruit Company have used without laboratory control enemas of potassium permanganate in the treatment of lobar pneumonia with more or less favorable results which, however, indicate no specific action of the agent used. The same may be said for sodium salicylate as reported by Couvey and Popoff.[285] Hexamethyltetramine (Urotropin) was found by Maeji[851] to be capable in a concentration of 1 to 1,000 to 1 to 2,000 of killing pneumococci *in vitro*.

Any further recital of reports on other therapeutic agents would add nothing of value to the present discussion, which may now be directed to a class of substances possessing markedly specific antagonistic properties against pneumococci.

* Quoted by Neufeld and Schnitzer.

Cinchona Derivatives

EFFECT OF THE VARIOUS QUININE DERIVATIVES ON PNEUMOCOCCI

Quite different from the agents already described in their action on pneumococci are certain products prepared from the alkaloid, cinchona. In the first experiments reported by Morgenroth and Levy (1911),[926-7] quinine was found to be valueless in sparing from infection mice inoculated with a fatal dose of pneumococci. However, when the related substance, hydroquinine (methylhydrocupreine) was used, the pneumococcidal effect was marked and a yet more pronounced action was observed when the next higher homologue, ethylhydrocupreine (optochin) was employed. The two substances in the form of aqueous solutions of their respective sulfates and hydrochlorides, when injected into mice at the time of inoculation or during the course of a virulent pneumococcal infection, saved a significant number of the test animals. Ethylhydrocupreine sulfate was less toxic and more potent in protective and curative action than was the hydrochloride of methylhydrocupreine. Another homologous derivative possessing active pneumococcidal properties, as determined by Morgenroth and Bumcke,[921] was isopropylhydrocupreine. Vuzine, described by Morgenroth,[920] is also an active homologue, but its action is influenced by variations in the susceptibility of different pneumococcal strains.

A disadvantage of the watery solutions of these alkaloidal salts was their rapid absorption and excretion—a disadvantage overcome by Morgenroth and Kaufmann,[922-5] as well as by Gutmann,[582] by dissolving the optochin base in oil. The toxicity of this type of compound, according to Morgenroth and Kaufmann, could be lowered by substituting the salicylic ester of optochin, and it was found that of the homologues of hydroquinine, optochin was the most active in germicidal effect on pneumococci.

The sterilizing action of optochin on pneumococci *in vitro* was demonstrated by Wright, Morgan, Colebrook, and Dodgson,[1543-4] a result corroborated by Schiemann and Ishiwara.[1230] Optochin in

concentrations of about one to one million and somewhat less inhibited the growth of pneumococci, while its bacteriostatic and pneumococcidal action was exerted in solutions of high protein content. The fact is of importance in relation to the effect of optochin on pneumococci in the blood stream—an effect demonstrated by Wright, Morgan, Colebrook, and Dodgson, as well as by Kolmer and Borrow.[742] The experiments of Moore[908] showed the highly specific action of ethylhydrocupreine hydrochloride on pneumococci and, moreover, disclosed the fact that the phenomenon took place with pneumococci of all the four groups tested. So specific was the effect that Moore saw in it a means for the identification of pneumococci.

The preeminence of ethylhydrocupreine among the quinine derivatives was established by Tugendreich and Russo,[1429] an observation confirmed by Solis-Cohen, Kolmer, and Heist[1304] among others. Felton and Dougherty[422] made a comparative study of four closely related synthetic cinchona derivatives. The first preparation was hydroquinine chloracetanilide, and the remaining three were substitution products of the first compound. The effect of the preparations was tested *in vitro* and *in vivo* against a young culture of Type I Pneumococcus. The *in vitro* tests were carried out by holding the dose of the drug constant in mixtures with varying amounts of culture, and then by injecting uniform amounts of the organism and increasing doses of the drug. In the *in vivo* experiments, a fatal dose of the culture was injected and the medicament administered in the maximal tolerated dose. All four compounds tested were found to display rapid pneumococcidal activity both in the test tube and in the peritoneal cavity of mice and to a lesser extent in rabbits. Optochin appeared to be the slowest in action, but its action was not so easily inhibited either *in vitro* or *in vivo*. The *meta* substitution product (hydroquinine *m*-chloracetylaminophenol) exerted the strongest killing action on pneumococci when injected simultaneously with the culture into the peritoneal cavity. However, optochin, notwithstanding the fact that the zone be-

tween therapeutic dose and toxic dose was narrower than in the case of the other derivatives studied, appeared to be less injurious to the natural defenses of the body.

Felton and Dougherty noted variability in the bactericidal action *in vivo* of the compounds on different strains of pneumococci, since the virulence of the culture contributed to fluctuations in the effect of the drug. The observation was contrary to those of Kleinschmidt,[722] of Moore, and also of Solis-Cohen, Kolmer, and Heist, whose results indicated that the germicidal power of the cinchona derivatives which they studied, possibly with the exception of some Type III strains, was apparently the same for all the types of pneumococci tested. Schiemann, and Schiemann and Ishiwara, however, had earlier reported that virulent strains were more susceptible to the effect of optochin than were attenuated strains, and that the source of the culture was also concerned in sensitivity to drug action.

According to Wright, Morgan, Colebrook, and Dodgson, and to Schiemann and Ishiwara, optochin, unlike some metallic salts, loses little of its germicidal power in the presence respectively of human serum or rabbit serum, but Solis-Cohen and his associates reported that the agent lost from one-fiftieth to one-tenth of its activity in a menstruum of serum as compared with that exhibited in isotonic salt solution.

Wright and his co-workers had early found (1912) that three hours subsequent to the administration of optochin to adults the serum of the treated subjects would kill pneumococci *in vitro*. Moore and Chesny[910] later made the same observation but stated that the effect depended upon the administration of a sufficient amount of the drug and the spacing of the doses. Similarly, Baldwin and Rhoades[69] concluded that the ethylhydrocupreine base, as employed by them, is absorbed from the gastro-intestinal tract in

sufficient quantities to impart demonstrable pneumococcidal prop-
erties to the serum when tested *in vitro*.

In carrying out pneumococcidal experiments with derivatives of
cinchona, the technique, the temperature of the mixture, the dura-
tion of exposure, and the vigor and virulence of the organisms em-
ployed should be considered and should always be uniform. Solis-
Cohen and his colleagues advised for the best results a small
number of organisms and a long exposure to the action of the
drug. Kolmer and Idzumi,[748] in studying the effect of optochin on
Type II pneumococci in purulent spinal fluid, found that steriliza-
tion was complete in sixty to ninety minutes when the preparation
was used in a concentration of 1 to 10,000 but, in a concentration
of 1 to 50,000, optochin was ineffective. In a more highly purulent
empyema exudate containing Type I Pneumococcus, Kolmer and
Sands[749] noted death of the organisms after a thirty-minute ex-
posure at 37° to an equal volume of optochin in a 1 to 10,000
solution. Maeji[852] gave the following figures as representing the
effective pneumococcidal strengths of the cinchona derivatives
tested: optochin hydrochloride, 1:8,000 to 1:10,000; remijine,
1:8,000 to 1:10,000; quinine, 1:2,000 to 1:4,000; eucopine,
1:20,000 to 1:40,000; vuzine, 1:30,000 to 1:40,000. Mention of
the curative action of the compounds was not included in Maeji's
papers.

A more delicate method for determining the bacteriostatic ac-
tion of optochin was that described by Schnabel.[1238, 1240] The re-
ducing property of the bacterial cell was chosen as an indicator of
the vital activities of Pneumococcus and the effect studied of the
drug on the ability of the organism to reduce methylene blue.
Schnabel reported that optochin in as low a concentration as one
to eight million was able to inhibit the reduction of the dye by the
strain of the Pneumococcus chosen for the test. The reaction could
also be utilized to determine the amount of optochin in the serum
of animals treated with the drug.

More recent experience casts doubt on the effectiveness claimed for optochin in combating pneumococcal infection. Reimann and Moen,[1131] for example, employing the Goodner intradermal method for infecting rabbits, after comparative trials with large doses of quinine hydrochloride and ethylhydrocupreine in rabbits, concluded that the feeble curative action of the drugs was in no way comparable to the effect produced by specific immune serum. The skepticism felt in Germany concerning the work of the American authors led Gundel and Seitz[577] to investigate several hitherto untried quinine derivatives. As a result of their experiments on mice, the authors placed ethylapoquinine in the foremost rank. The compound in high dilution killed pneumococci in fairly large numbers both *in vitro* and *in vivo*, and in its action was to be preferred to *alpha* and *beta*-isoquinine and even to optochin.

In contrast to the report of Gundel and Seitz was that of Kolmer and Rule[748] who, like Reimann and Moen, tested cinchona preparations on rabbits suffering from "dermal pneumonia" following inoculation by the Goodner technique. Neither the optochin base, ethylhydrocupreine hydrochloride, nor quinine and urea hydrochloride, given by stomach tube and by repeated intramuscular injection, exhibited any beneficial effect on the local lesion, bacteriemia, or leucocyte count resulting from intradermal infection with Type I pneumococci. In a more extended study comprising tests on some thirty-five preparations of the quinine group, Maclachlan, Permar, Johnston, and Kenney[846] found that only three compounds of those tested possessed any conspicuous killing power for pneumococci, and these substances were ethylapoquinine, hydroethylapoquinine, and hydroxyethylhydrocupreine. The authors' preference was given to ethylapoquinine because it gave greater protection to the mice treated than did optochin. The results were not in agreement with those of Okomoto and Sogen,[1024] who found that while apoquinine exhibited strong pneumococcidal action *in*

vitro, it afforded practically no protection to mice against experimental pneumococcal infection.

Other experiments that serve to substantiate the claims of Maclachlan and his colleagues are those of Butler, Nelson, Renfrew, and Cretcher.[193] The authors tested cinchona alkaloids, their hydrogenated derivatives, and some artificially prepared homologues and reported that hydroxyethylhydrocupreine was less toxic for mice than optochin and was highly efficient in protecting the test animals against pneumococcal infectio . In a second communication, Butler and Cretcher[192] stated that apocupreine hydrochloride displayed fairly high pneumococcidal action *in vitro*, when compared to other members of the cinchona group, was very low in toxicity for mice, and possessed protective properties similar to those of optochin and ethylapoquinine.

Optochin and its allied compounds possess marked disadvantages. The substances do not penetrate the infected lesions and, therefore, do not affect the cocci at the point of greatest concentration. A more serious drawback is the toxic action of some of the drugs of the cinchona series. Moore and Chesny,[910] in an analysis of the manifestations observed during optochin treatment of lobar pneumonia in man, pointed out the well-known tendency of optochin to damage eyesight. The authors stated that in the literature on optochin treatment, among the patients developing serious ophthalmic disturbances with impairment of vision (4.5 per cent of those reported) there were some whose iris failed to react to light and others in whom the ophthalmoscope revealed a tortuosity of the retinal vessels with a general constriction of the arteries. Acute retinitis accompanied by temporary blindness has been observed, but interference with vision is rarely permanent. Browning[159] has stated that this complication has occurred mainly after administration of the soluble hydrochloride and may be minimized by avoiding excessive doses or by the use of relatively insoluble forms of the drug. However, the comparative insolubility of the base

constitutes a disadvantage in its use. A precaution to be observed in this class of substances is to keep the compound from coming in contact with free hydrochloric acid in the stomach.

ADJUVANT ACTION OF CINCHONA COMPOUNDS WITH SPECIFIC
SERUM

The first report on the combined action of optochin and immune serum on pneumococci was that of Neufeld and Engwer[982] in 1912 and of Engwer[365] in 1913, who found in guinea pigs an augmented action of the two agents when employed together. Another observation reported by Boehncke[132] in 1913 suggested that optochin may exert an auxiliary or possibly a synergistic action when administered along with specific immune serum. In tests on white mice, Boehncke[133] claimed better curative effects with a mixture of ethylhydrocupreine and immune serum than with either agent alone and that this favorable therapeutic action could be produced by the combined use of both agents in less than the curative dose of either. The observation was confirmed by Moore,[909] who concluded that a single small dose of optochin base, which by itself exerted no protective action against pneumococcal infection in mice, was capable of increasing by at least fifty times the threshold value of antipneumococcic serum. The effect occurred only when the serum and the infecting organism were of the same serological type and proportionally was many times greater than a simple summation of the protective action of the two single components of the mixture.

The phenomenon finds an analogy in the adjuvant action of sodium oleate with immune serum already quoted from Lamar[775] and that of gold as reported by Gelarie and Sabin.[510] The use of cinchona derivatives, especially ethylhydrocupreine, in combination with specific serum therapy would appear to possess the advantage of making it possible to reduce the dose of the drug well below the toxic limit as well as to enhance the curative action of the immune

serum. Search has failed to reveal any record of further trials of this combined form of treatment in pneumococcal infections.

In this connection a comparison of the protective power of cinchona derivatives and of antipneumococcic serum may not be out of place. Felton[404] reported that the specific protective globulin of immune horse serum was one million times more effective against Pneumococcus than was quinine sulfate and one hundred thousand times more powerful than optochin.

EFFECT ON VIRULENCE OF PNEUMOCOCCUS

Drug-fastness. In Chapter V, the effect of optochin in inducing variation in pneumococci was described. In that biological change the bacterial cell acquires a tolerance for the drug. Morgenroth and Kaufmann[922-5] showed that the passage of a pneumococcal strain four times through animals treated with optochin caused the organisms to lose their sensitivity to the action of the drug. The observation was confirmed by Tugendreich and Russo,[1429] and by Koehne,[738] who discovered that pneumococci suffered an enormous loss of susceptibility after cultivation in the presence of optochin *in vitro*. Schnabel and Jungeblut* likewise demonstrated the acquisition by the bacterial cell of a high-grade resistance following cultivation in broth containing optochin. Koch, by growing pneumococci in artificial media, could decrease optochin sensitivity without destroying the virulence of the organisms. After a few animal passages the strains regained their normal sensitivity.

During this process of biological adaptation, according to Jungeblut,[697] the bacterial cell may lose virulence but, on the other hand, Lewy[807] was able to maintain the full virulence of strains of pneumococci by alternate passage through untreated mice and through culture media containing optochin, although the strain had become eighty to one hundred twenty times more resistant to

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* Quoted from Neufeld and Schnitzer,[1000] from whose account and that of Browning[159] much of the above information is taken.

the chemical. Drug-fastness was retained for some time during
cultivation in optochin-free media as well as during animal pas-
sage. Lewy noted that during the treatment just described vari-
ants of the original strain appeared (Modification B) which
lacked sensitivity to optochin. The resistance or tolerance was
general in nature and was not to be confused with specific opto-
chin-fastness. In an analysis of the specificity of optochin-fastness
in pneumococci, Lewy[808] found that there was a slight, specific ac-
tion of quinine as well as of hydroquinine. With more marked
chemical variations in the side-chain, as in the case of the higher
homologues, or in the nucleus of the optochin molecule, the spe-
cific action of optochin on Pneumococcus disappears. The sus-
ceptibility of the optochin-fast strain to other compounds quite
foreign to this class of substances suggested to Lewy the presence
of other sensitive groups—chemoceptors—in the pneumococcal
cell.

Heightened susceptibility. That the reverse of acquired toler-
ance by Pneumococcus may take place was shown by the experi-
ments of Schnabel[1241] and of Schnabel and Kasarnowsky.[1242] Cul-
tures of pneumococci injected into mice with subsequent adminis-
tration to the infected animals of dilute optochin solutions caused
the organisms after a twenty-four-hour sojourn in the body of the
treated animals to develop a heightened susceptibility to the drug.
In addition to the influence of lesser concentrations of optochin,
the length of exposure of the organisms in the treated animals was
a factor in increasing the sensitivity of the cocci.

Summary

In reviewing the data which have been accumulated from experi-
ments in the test tube and in the animal body, the conviction is
gained that not yet has the ideal drug been found that lends itself
to the chemotherapy of pneumococcal infection. The action of cin-
chona derivatives in affecting the vital characters of Pneumococ-

cus may render the bacteria more vulnerable to the natural defensive forces of the body, and this action may be a distinct aid in specific serum therapy. Failure so far to discover a satisfactory medicinal agent with pneumococcidal properties and free of undesirable side effects is tempered with the promise offered by cinchona compounds and leaves this inviting and profitable field open to renewed and extended investigation.

NOTE: The introduction of the sulfonamide derivatives (sulfanilamide and more especially sulfapyridine and sulfathiazole) has completely altered the aspect of chemotherapy. The very general use of these drugs and their recognized effectiveness need only be mentioned to establish this point.

E. S. R.
L. A. B.

March, 1941

PRODUCTION OF ANTIPNEUMOCOCCIC SERUM

Procedures employed in the routine manufacture of therapeutic serum, including immunization of horses, methods of concentration and purification of specific immune serum; processing, standardization, and control of the products; and the preparation of serum for diagnostic purposes.

THE early demonstration that serum from immunized rabbits and other small experimental animals exerts protective and curative effects in similar animals infected with pneumococci supplied ample warrant for extending studies to include larger domestic animals in the hope of providing serum for use in the treatment of pneumococcal infection in man. Interest in this phase of study first developed by 1897, for in that year there appeared publications by Denys,[312] Pane,[1044] Mennes,[893] and Washbourn[1487] describing the results attending the immunization of goats, asses, cows, and horses. Occasional reports on the use of other animals have been made, but where large amounts are required horses are at the present time the commonly accepted source of antipneumococcic serum.

Immunization of the Horse

SELECTION OF HORSES

Little is known as to the precise type or breed of horse most suitable for the production of antipneumococcic serum. Avery, Chickering, Cole, and Dochez[36] preferred sound, fairly heavy horses, and the same opinion is shared by some laboratories today. At the Massachusetts Antitoxin and Vaccine Laboratory experience has shown that the breed, weight, and sex of horses are less important factors than the variations in the immune response of

individual animals. Not infrequently it has been observed that a horse showing poor response to injections of diphtheria and scarlet fever toxins or even of suspensions of Meningococcus may develop a high titer of specific humoral antibodies following immunization with pneumococcal vaccines. It is equally true that animals of the same general type may produce serum exhibiting considerable differences in potency. The trial-and-error method of selection of horses for immunization, while expensive and time-consuming, appears to be the most reliable procedure available at the present time. There is an impression in certain quarters at least, that the age of the horse may be of some importance. Wadsworth and Kirkbride[1470] used horses of over eleven years of age. Although it has not been proved that older horses are more readily immunized with pneumococcal vaccines, it can be said that the more mature horses respond quite as well as do younger animals and, furthermore, are likely to be more tractable.

Prior to initiation of the immunization process, the horses selected should be subjected to a rigid physical examination, with particular attention to the existence of respiratory affections. Animals exhibiting evidence of the "heaves" should not be used. A quarantine period of three weeks should be the rule, during which time a careful examination for glanders by physical means and by the mallein and complement-fixation tests is made, and the absence of other communicable diseases determined. When the animals have satisfactorily passed the quarantine period they may be stabled in permanent quarters and the immunization process begun.

SELECTION AND STANDARDIZATION OF THE IMMUNIZING ANTIGEN

The ideal pneumococcal antigen for the routine immunization of horses is yet to be found. Such questions as the degree of virulence of the cultures, whether the organism should be in a living or devitalized state (or combinations of both), and which derivatives of the pneumococcal cell possess the desired antigenic properties,

along with a host of modifications in the preparation of each anti-
gen have been considered in attempts to increase the therapeutic
potency of antipneumococcic serum.

Virulence. The virulence of strains of pneumococci used for im-
munization was recognized as an important factor soon after im-
munological studies were begun. Emmerich and Fowitzky (1891)[357]
found that rabbits injected subcutaneously with attenuated cul-
tures became only partly immune, but after the intravenous admin-
istration of fully virulent cultures a "complete" immunity resulted.
It should be borne in mind, however, that while the difference in the
routes of injection may have played a part in the outcome of the
experiments, the conclusions reached are in accord with present
conceptions. A year later, Mosny[932] stated that cultures used for
immunization should be virulent, and added the requirement that
cultures should be heated for three hours at 60°. Emmerich,[356] and
Römer[1155] both subscribed to the idea of using only virulent strains
for immunization, and Landmann,[779] who worked under Römer,
found that more uniform and potent serums were obtained when
only highly virulent strains of pneumococci were employed. Neu-
feld and Haendel[989] also used only strains of exalted virulence.
Wadsworth,[1458] in determining the curative and protective activity
of immune serum in infected rabbits, reported that while immune
serum might be protective, only after immunization of animals
with living, virulent cultures did the serum acquire marked cura-
tive properties. In the same year, Dochez[316] reported the develop-
ment of highly potent protective serum by immunization of the
horse with large doses of virulent organisms in the living state.

In the well-known Rockefeller Monograph No. 7, Avery and his
colleagues[36] emphasized the importance of using virulent strains
for immunization. So, too, Raphael[1121] declared that the degree of
immunizing action is dependent upon virulence, and advocated a
study of the antigenicity of only virulent strains in rabbits before
attempting the hyperimmunization of larger animals. In studying
the response of mice and rabbits to immunization with living or

killed avirulent pneumococci, Yoshioka (1925)[1565] found that only weak serums were produced. During investigations on the smooth and rough forms of pneumococci, Griffith (1923)[560] concluded that only smooth strains should be employed for the production of serum. Using Type III pneumococci, Lévy-Bruhl (1927)[804] obtained agglutinating and precipitating serums readily from horses, but protective antibody was obtained only when recourse was had to the injection of exceptionally virulent strains. Meyer and Sukneff (1928)[898] reported that the administration of sodium taurocholate solutions of virulent strains yielded serum with protective and some cross-protective powers, but the injection of solutions of avirulent strains evoked no protective antibodies. The observations are consistent with the earlier reports of Avery and Neill[59] and of Avery and Heidelberger[49] on the antigenic action of constituents of the pneumococcal cell. In studies on the response of rabbits to inhalation of pneumococci, Stillman (1930)[1334] concluded that the type-specific response of the animals, as evidenced by the appearance of specific protective antibodies and agglutinins in the serum, varies in direct proportion to the virulence of the culture used. There seems to be little dissent, therefore, from the opinion that only cultures of maximal virulence furnish satisfactory antigenic material for the production of potent antipneumococcic serum. In the opinion of the authors of the present volume, the best results are obtained by choosing cultures of exalted virulence and strong antigenicity and by maintaining high virulence by passing the strains through mice daily for at least five days a week. The various methods of maintaining the virulence of cultures in routine use have been discussed in Chapter VI.

In a study of the immunizing properties of the four groups of pneumococci classified by Dochez and Gillespie,[322] Dochez and Avery[318] found the order of virulence of the types of pneumococci for human beings to be III, II, I, and Group IV, and stated that "the degree of protective power developed in the sera of animals immunized against members of these groups varies inversely with

the virulence and the amount of capsular development." The statement of Dochez and Avery just cited should not be construed as meaning that the virulence of a strain of Pneumococcus militates against its ability to provoke the elaboration of immune substances in the animal body. There are other considerations peculiar to each pneumococcal type that determine the antigenic properties of its members. The factors may be differences in the chemical constitution of the cellular components or in the manner in which the polysaccharide and other elements of the cell are joined together, which affects the lability or stability of the antigen when introduced into the body and, therefore, determines the specific immune response of the animal. Thus, it is known to be more difficult to obtain a potent serum for Type III than for Type II Pneumococcus, and easier to produce serum of higher protective value for Type I than for Type II. Investigations on the twenty-nine separate types formerly in Group IV indicate differences in antigenicity which may follow the principle established by Dochez and Avery. Attempts to produce serum effective against Type III pneumococci, possessing as they do an unusually large amount of capsular material, have been disappointing.

Filtrates of broth cultures. Much of the early work was done with filtrates of broth cultures or other agents containing the soluble, cell-free components of the pneumococcal cell. Of great importance are the investigations by Avery and Morgan (1925),[54] Avery and Neill (1925),[59] and Avery and Heidelberger (1925).[49] The results of these studies show that solutions or extracts of Pneumococcus containing free somatic protein and free capsular polysaccharide give rise only to species-specific antibodies; suspensions containing mixtures of intact cells and dissociated cellular components induce both species-specific and type-specific antibodies; preparations of intact cells containing no free pneumococcal protein stimulate the production of type-specific immunity. Confirmation of these findings came from Barach,[75] Meyer and Sukneff,[898] Ferguson,[436] and from various unreported observations

made during the routine work in manufacturing laboratories. At present, therefore, the most satisfactory antigen for stimulating the production of type-specific therapeutic serum is one composed of a natural combination of the pneumococcal protein and polysaccharide as found in the intact cell, and containing a minimum of uncombined carbohydrate and somatic protein.

Exudates of infected tissues. Exudates of infected pneumonic tissue have also been tried as immunizing agents. Netter (1887)[969] obtained immunity in mice and rabbits by the injection of post-pneumonic pleural exudate, and the Klemperer brothers (1891)[724] observed similar results. Hartman (1913)[599] also described the use of the various constituents of exudative material as antigens but the results were disappointing. A vaccine prepared from pneumococci washed from the peritoneal cavity of a rabbit dead from pneumococcal infection was, according to Heist and Solis-Cohen (1919),[635] more active as an immunizing agent for rabbits than one made from the organisms grown on artificial culture media. Freedlander (1928),[480] by using saline extracts of infected tissues as antigens in rabbits, claimed to have obtained protective serum of a potency corresponding to that of antipneumococcic horse serum. In 1929, Curphey and Baruch[291] described a method of collecting and using pleural exudate from horses in the immunization of other horses against pneumococci. The authors believed that the procedure would decrease the length of time necessary to produce a potent immune serum. A year later, these investigators[292] reported the results of tests with the so-called exudate antiserum. The effects of the serum in treating intradermal infections in rabbits were compared with those of serum prepared in the usual manner, and in the opinion of the authors the exudate antiserum contained antibodies other than those of an antibacterial nature.

Viability of the organism. The question whether dead pneumococci are more or less efficient antigens than living pneumococci is still unsettled. Mosny (1892),[932] already quoted, stated that when cultures are to be used for immunization, "they must be virulent

and heated for three hours at 60°," thus favoring the use of killed suspensions. Issaeff[673] reported successful immunization of rabbits by administering killed bouillon cultures and later blood from infected animals, the organisms being first killed by chloroform or heat. In immunizing horses, Denys (1897)[312] injected first heated, then unheated cultures, next the blood of a rabbit dead of pneumococcal infection, and finally living broth cultures. He thus was able ultimately to inject a large amount of an extremely virulent culture without causing any more marked symptoms than a transient rise in temperature. Mennes (1897),[893] by using methods similar to those of Denys, produced in the horse after three or four months' treatment, serum that exhibited preventive, curative, and "antitoxic" properties. Washbourn (1897)[1487] immunized ponies by the subcutaneous injection of heated broth cultures, then living agar cultures, and finally living broth cultures. After nine months of treatment, the serum was found to possess marked protective action.

Neufeld (1902)[974] recommended the use first of killed, then of living organisms. Later, however, with Haendel (1909),[989] Neufeld stated that in immunizing horses and asses there was no such marked difference between the use of living and dead organisms as was found in rabbits, although horses injected with viable cultures produced better serum. Horses proved to be more sensitive than asses to living organisms.

For developing antipneumococcic serum of high protective titer in horses, Dochez (1912)[316] employed large doses of living, virulent organisms. In an investigation of the action of immune serum on pneumococcal infection in rabbits, Wadsworth (1912)[1458] found that little or no curative effect was exerted by serum from animals immunized with washed, dead organisms or culture filtrates; only after immunization with living, virulent cultures did the serum acquire marked therapeutic properties. In a study on the distribution of immune bodies in antipneumococcic serum, Avery[32] immunized horses over periods of one or two years by in-

jecting progressively increasing doses of dead organisms until the animals were able to tolerate the sedimented residue from one liter of living, virulent culture. Cole and Moore (1917)[268] also favored this method. Their experiments indicated that, for the most rapid production of primary immunity, several series of small doses of dead cultures should be given, but in order to produce the highest degree of immunity, living organisms were required.

In the Rockefeller Monograph, Avery, Chickering, Cole, and Dochez[36] outlined their methods for the immunization of horses. After the administration of two series of injections of heat-killed organisms, a sample of blood was obtained for agglutination and protection tests. Serum taken at this time was never found to be of satisfactory titer and, consequently, injections of living organisms were given in increasing amounts until tests on the serum samples indicated the desired potency. Cole (1917)[257] obtained potent serum from horses injected over a period of six to seven weeks with gradually increasing doses of dead organisms. Wadsworth and Kirkbride (1917)[1470] reported the development of protective serums in horses immunized first by injections of heated organisms followed by increasing doses of living pneumococci. In 1918, Alexander[7] described the use of mixtures of pneumococci and leucocytes, and of serum-sensitized organisms and leucocytes, in immunizing rabbits, and found that protective antibodies were developed within eight to eleven days.

Despite the observations and beliefs that living cultures of pneumococci induce the production of more effective therapeutic serum than do killed organisms, there has been an increasing tendency to use dead cultures. It is doubtful if any of the larger manufacturing laboratories employ living cultures for the routine immunization of horses. The writers of the present volume question whether the alleged superiority of living antigens over devitalized preparations in stimulating antibody production outweighs the hazards associated with the use of living, virulent cultures. Granting the opinion to be sound, the problem arises of choosing a method by

which suspensions of pneumococci may be rendered non-infective but which will preserve a maximum of antigenicity.

Methods of devitalizing the organism: heat and formalin. Heat has long been employed as a means of devitalizing organisms to be used for immunization purposes. Mosny[932] recommended the heating of cultures for three hours at 60°. Washbourn[1487] advocated a temperature of 60°, but for a period of only one hour, and Neufeld and Haendel[989] also utilized the same temperature. In preparing killed suspensions of pneumococci, Avery, Chickering, Cole, and Dochez[36] heated the coccal suspensions in the water-bath at 56° for one-half hour. Truche (1920)[1420] preferred vaccines killed by alcohol and ether, since he stated that heat-killed suspensions were not tolerated well by horses. In a study of the effect of the degree and period of heating on the antigenicity of pneumococci, Tani[1379] obtained better immunization with vaccines subjected to 100° than with those heated at lower temperatures. The period of heating varied from ten minutes to four hours. A culture heated at 45° for two and one-half hours was found to have low immunizing value. In the investigations of Gaspari, Sugg, Fleming, and Neill,[503] in which a large number of rabbits were used as test animals, the influence of heat on the antigenic strength of the type-specific and species-specific antigens of the Type II pneumococci was studied. The temperatures varied from 55° to 120° applied for a period of one-half hour, and the results indicated that heating pneumococcal suspensions tends to decrease the effectiveness of the species-specific antigen without having any appreciable influence on the antigenic power of the type-specific complex.

Chemical agents have also been employed in preparing pneumococcal vaccines for animal immunization. The use of alcohol and ether has already been mentioned, while glycerol extracts have also been tried. Takami[1374] used carbolized cultures with rabbits, but there appear to be few adherents to the practice. By the subcutaneous injection of horses with ricinoleated cultures, or with sterile pneumococcal "toxin," Clowes, Jamieson, and Olson[244] reported

the demonstration of antitoxic properties in immune serum so produced.

The methods of choice in devitalizing pneumococcal vaccines appear to have become reduced, in recent years, to treatment either with heat or with formalin. For immunizing horses, Savino, Negrete, and Acuna (1931)[1221] added 0.2 per cent formalin to cultures and then centrifuged out the organisms. Pico and Negrete[1089] added 0.5 per cent of formalin to suspensions to be used for the same purpose, and claimed that the preparations retained their antigenic power over a long period of time. Tao[1380] compared the immune response of rabbits and mice injected with heat-killed vaccines and suspensions of pneumococci killed by exposure to 0.3 per cent formalin for eight hours. He reported that formalinized vaccines stimulated the production of specific antibodies quite as well if not better than did heated suspensions, but it was observed that the immunity developed in the animal body under the stimulus from formalin-treated vaccine appeared somewhat later than that from heated vaccine. The latter observation was confirmed by the work of Barnes and White (1934)[86] during studies on the comparative antigenicity of heat-killed and formalinized vaccines in rabbits and horses but, contrary to Tao's observations, vaccines killed by one-hour exposure to 56° were at least as effective, if not more so, than the formalinized suspensions. In addition, heat-killed vaccines appeared to be somewhat less toxic than those treated with formalin.

The amount of formalin used by different workers in preparing pneumococcal vaccines has varied. In this connection, some observations by Barnes and Hager* (1932) may be cited. A culture of pneumococci was divided into four parts and to each portion there was added 0.2, 0.3, 0.4, and 0.5 per cent respectively of formalin. After storage at room temperature overnight, the cultures were centrifuged and the supernatant fluids tested for free and combined formaldehyde. In each case only about 0.05 per cent of for-

* Unpublished.

malin remained in combination with the sedimented organisms. This fact implies that when formalin is added in a concentration greater than that necessary for devitalizing pneumococci, the excess may remain uncombined, and participate in some of the disturbing reactions in animals injected with formalinized vaccines.

Results of investigations by Barnes and Wight* (1935) suggest that formalinized suspensions of pneumococci, particularly those of Type II, undergo rapid autolysis during storage in the refrigerator; vaccines killed by heat at 56° for one hour are somewhat more stable, while those heated at 100° for fifteen minutes retain their original turbidity for periods of at least four months. The antigenic potency, in rabbits and horses, of pneumococcal vaccines devitalized by heat at 100° appears to be as high, if not higher, than that of the more readily autolyzable preparations.

In the authors' experience, pneumococcal vaccines prepared by heating the cultures at 100° for fifteen minutes have yielded satisfactory results in the immunization of rabbits and horses and have been adopted in the routine immunization of the latter animals for the production of antipneumococcic serum.†

Dried antigens have also been employed, largely on an experimental basis, but the preparations have not been used routinely to a sufficient extent to warrant detailed description.

Standardization. Regardless of the method used in preparing pneumococcal antigens for the routine immunization of animals, it is desirable to standardize the vaccine. In order to obviate laborious and time-consuming bacterial counts on each preparation, many of the standardization methods have depended upon a comparison of the density of the bacterial suspensions with that of suspensions of inert materials such as silicates, fuller's earth, barium sulfate, and so forth, in which a given amount of the material chosen is made to correspond to a given bacterial count.

Gates[504] contrived a simple device for determining opacity by measuring the depth of disappearance of a wire loop when im-

mersed in a column of bacterial suspension. A formula was derived by which the reading obtained can be translated into terms representing the concentration of bacteria per cubic centimeter of fluid. Once this figure is determined for the suspension chosen as a standard, it is necessary only to adjust each new preparation of vaccine to a density affording corresponding readings by the instrument.

By a method similar to the older technique of Hopkins, Krueger[760] measured the cell concentration of bacterial suspensions and reported a deviation of 2 to 5 per cent from actual counts. Feemster, Wetterlow, and Cianciarulo[393] recently compared the values obtained with suspensions of typhoid bacilli by direct counts, nitrogen determinations, and the Gates densitometer readings and found the latter two methods to be highly correlated. Since the nitrogen content is an index of bacterial mass, rather than of numbers, and hence presumably is related directly to antigenic value, determinations of density by the Gates apparatus appear to afford a simple and satisfactory means of measuring antigenic substance and therefore of standardizing bacterial vaccines. A corrected Gates reading of 2.0 centimeters on a 1 to 30 dilution of suspension of pneumococci corresponds approximately to 25 billion organisms per cubic centimeter, which furnishes a satisfactory standard suspension from which measured doses of vaccine may be made for routine injections.

INJECTIONS

Route of injection. As early as 1891, the Klemperer brothers[724] introduced the intravenous method of injecting pneumococcal preparations, having found the immunological response to be superior to that following subcutaneous injection. For stimulating the production of pneumococcal antibodies, the intravenous route for the injection of antigen is the method of choice. In the search for therapeutic serum of greater efficacy, it may prove of value to administer antigenic materials subcutaneously with the hope of

stimulating the production in the horse of antibodies in addition
to those commonly found in immune serum. For the purpose, the
agar-agar technique of Dochez* (1924), the blood-clot method
of Zinsser and Grinnell,[1580] or the procedures followed by Clowes,
Jamieson, and Olson,[244] or those of Parker and McCoy[1062] may be
useful.

Dosage. It is difficult to evaluate many of the earlier reports re-
garding the dosage of antigens used, since the descriptions re-
ferred only to the volume of cultures, extracts, solutions, or other
preparations of pneumococci, without any indication of the bac-
terial content. There is an optimal range in the amount of vaccine
which will induce satisfactory antibody production without caus-
ing harmful effects in the animal. Landmann (1908)[779] observed a
greater loss of horses following increases in dosage. When living
cultures are used for immunization the doses cannot be increased
as rapidly as when killed antigens are used (Neufeld and Haendel,
1909[989]). Avery and his associates[36] stated that "doses should be
small, as very large doses inhibit the immunity response." The au-
thors recommended that the heat-killed suspensions used for injec-
tion should contain about 200 to 300 million organisms per cubic
centimeter and the dose should represent the bacteria from 50
cubic centimeters of culture; with living organisms, the first injec-
tion consisted of the organisms from 2.5 cubic centimeters of
original culture. Doses were usually doubled progressively if the
reaction of the horse was favorable until the maximal dose consist-
ing of the sediment from 300 to 400 cubic centimeters of broth
culture was administered.

In a study of the immune response of rabbits to prolonged im-
munization with vaccines of Type I Pneumococcus, Barnes and
White[86] determined the density of the bacterial suspensions as a
measure for regulating dosage. Based on the experience thus
gained, the present practice of the authors in immunizing horses is
as follows: All vaccines are standardized by means of the Gates

* Personal communication.

apparatus to a corrected reading of 2.0 centimeters in a 1 to 30 dilution of the standard suspension—approximately twenty-five billion organisms per cubic centimeter. Formerly, the doses were given in gradually increasing amounts until "holding doses" of 9.0, 10.0, and 10.0 cubic centimeters, or a maximum of about 250 billion pneumococci, were administered. More recently, the doses have been decreased, so that the usual amounts are approximately one-fourth (2.0, 2.5, 2.5 cubic centimeters of the standard suspension) of those previously used. The content of protective substances of the serum produced under the present scheme is as great as, if not greater than, it was when larger amounts of vaccine were used. It must be noted, however, that because horses vary widely in their response to injections of bacterial suspensions it is occasionally necessary in individual instances to decrease the dose still further.

Spacing. The spacing of injections of vaccines is important. In earlier investigations, the antigens were usually administered at weekly intervals. Wadsworth and Kirkbride[1470] began the immunization process by giving injections either weekly or on three successive days. Cole[257] scheduled injections daily for seven days as a single series, with a rest interval of a week, followed by seven more daily injections. Cole and Moore[268] gave injections of dead organisms daily for six or seven days, with a rest of a week, then a series of three daily doses of live organisms with an interval of a week between each series. Avery, Chickering, Cole, and Dochez[36] recommended the following procedure: Six daily injections of heat-killed vaccines were given, followed by an interval of seven days, after which a second series of six daily doses was administered. Injections of living organisms were then made on three successive days, followed by a rest of seven days, when three consecutive daily doses were again given. This routine was followed throughout the course of immunization.

Using dried antigen, Truche[1420] gave injections on ten successive days and, after the lapse of from ten days to a fortnight, in-

jected increasing doses on four successive days, followed by a ten-
day rest period. In a comparison of results of different schedules
of injections of vaccines in rabbits, Yoshioka (1923)[1562] tried
three series of six daily injections at intervals of eight days, but
reported better results when the same dose was given in six aliquot
parts at one-half hour intervals in one day. Two years later
(1925), the same author[1565] reported the results of a more exten-
sive study in mice. One group of animals received three doses of
vaccine in a period of two weeks; a second was given injections
daily for three days; a third was injected six times at one-half-
hour intervals in one day; and the fourth group was given one
single injection. The total amount of antigen administered was the
same in each group. Yoshioka decided that the best results were
obtained by giving the vaccine to animals in divided doses at fre-
quent intervals, as in the third group. Killian,[706] however, failed to
confirm the observations.

Barnes and White[86] investigated antibody production in rabbits
receiving intravenous injections of formalinized and heat-killed
Type I pneumococci under three different schedules: 1) a single
injection on each of three successive days; 2) a single dose on each
of five consecutive days; and 3) a series of six injections, one on
each of the first two days with the other four at one and one-half-
hour intervals on the third day. Of the three plans, the first and
last yielded better results than the second, and the first was at
least as good as the last. The first scheme corresponds to the so-
called "three-week" schedule. In the routine immunization of
horses it has been found to give satisfactory results and is, more-
over, convenient. According to the three-week schedule of immuni-
zation, the following plan has been used at the Massachusetts
Antitoxin and Vaccine Laboratory: The horses are injected on
Tuesday, Wednesday, and Thursday of one week and on Thurs-
day, Friday, and Saturday of the following week. The animals are
bled on Monday, the ninth day after the last injection in the
course, and the same schedule of injections is resumed on the day
following the bleeding.

Whichever schedule of injections is chosen, it has been generally customary to allow a rest period of about a week between series. It is possible that a longer interval might be used to advantage, but it seems doubtful if shorter periods would be advisable. Barnes and White[86] observed that in rabbits in which a primary immunity had been established, rest periods of three weeks instead of the usual one week between courses of injections did not lessen antibody response to subsequent injections of antigen.

The experiments of Cole (1904),[248] of Hachtel and Stoner (1916),[583] and of Feemster (1932)[392] have suggested that antibody response of animals and man to typhoid vaccine is more rapid and vigorous in both animals and man who have either had the disease or been inoculated than in those who have never been in contact with the organism. The observations of Barnes and White[86] on rabbits under immunization against Pneumococcus bear on the question. The principle involved has been applied in the immunization of horses for the production of antipneumococcic serum. Unpublished experiments at the Massachusetts laboratory serve to illustrate the results that may be expected in immune horses when subjected to delayed secondary stimulation. A group of horses under immunization for periods sufficiently long to establish primary immunity were rested for approximately three months without receiving injections of any sort. Test bleedings on this group of horses, taken prior to the resumption of immunization, showed an average decrease of 75 per cent in antibody content during the three-month rest period. After the first course of six injections following the rest period, in which the total dosage of vaccine averaged about one-fifth that of the last course, there was found to have developed in the serum of the animals so treated an average increase in antibody content (as compared with titers prior to the rest period) of approximately 25 per cent for both Types I and II. The same principle seems likely to hold true if, instead of omitting all injections during the rest period, the horses are subjected to some other form of immunization. It is open to question, however, whether the increased potency of serum from horses after

such rest intervals is of sufficient value to counterbalance the loss of serum which might have been obtained had the animals been kept continuously on the routine schedule of immunization and bleeding.

Relation of materials injected to reactions in the horse. There is usually a febrile reaction in horses following each injection of pneumococcal vaccine. The rise in temperature varies with the individual animal and with the amount and kind of material injected. More severe reactions follow the administration of living cultures, dose for dose, than of killed suspensions. Thus, Avery, Chickering, Cole, and Dochez[36] referred to a body temperature of 40.5° as moderate, subsequent to the injection of living cultures. It is the custom in some laboratories to omit injections if the temperature of the horse under immunization remains above 38°. There appears to be no definite correlation between the temperature rise and the degree of antibody production, although an increase in body temperature may be related to immunological response to the substance injected.

There are reactions in horses undergoing immunization against Pneumococcus that are of a more serious nature. In some instances the animals exhibit symptoms of poor appetite and of gastrointestinal disturbances, or show abnormally high temperatures lasting for several days. Not infrequently, when the animals come to necropsy, extensive thrombus formation is observed, particularly in the blood vessels of the portal circulation. Occasionally, when respiratory disturbances are noted prior to death, thrombi are found also in the lungs. While it cannot be considered as proved, evidence has been accumulated to indicate that horses given intravenous injections of formalinized suspensions of pneumococci are more prone to develop thrombi than animals receiving vaccines devitalized by heat. In comparing the antigenic effect of heat-killed and formalinized cultures in rabbits, Barnes and White noted a smaller loss of animals in the former than in the latter group.

Another serious disturbance sometimes encountered in horses undergoing immunization is the occurrence of anaphylactoid reactions. The symptoms usually occur within a short time following an injection of vaccine and are characterized in typical cases by their abruptness, by excessive perspiration, marked respiratory embarrassment resulting in a blood-tinged, frothy exudate from the nostrils, complete collapse, and death shortly after the onset of symptoms. At necropsy, the gross pathological findings are usually negative except for marked congestion and hemorrhages in the lung tissue, and pulmonary edema. It is difficult to assign a definite cause to the reactions. A study of records at the Massachusetts laboratory showed that the majority of deaths of this nature occurred after the first injection in a series following the routine bleeding subsequent to the rest interval, and that deaths occurred most frequently in the period from two to eighteen months after initiation of the immunization process.

Because of these disturbances and the suggestion that the symptoms were indicative of a hypersensitive condition, the practice was adopted of administering subcutaneously a small dose of the vaccine on the day preceding the first of each series of three regular daily intravenous injections. These "desensitizing doses" were given to horses that exhibited unusual reactivity to the routine injections. Although the number of horses so treated has been too small to justify definite conclusions, the impression has been gained that the desensitizing treatment reduced the expected mortality rate of horses undergoing immunization. In the experience of the authors, the majority of sudden spontaneous deaths in horses have occurred when formalinized suspensions were in use and possibly may be referable, in part at least, to the more rapid lysis of these preparations as compared with vaccines killed by heating at 100°. Since the substitution of vaccines prepared in the latter manner it has not been found necessary to employ desensitizing doses. Truche[1419] claimed that death due to hypersensitivity could be avoided by diluting the organisms to be injected in a

large quantity of physiological salt solution. In order to combat these immediate reactions it is advisable to have on hand a supply of epinephrine which may be injected into horses showing symptoms of possible hypersensitivity; glucose solution may also be administered intravenously to advantage.

When living cultures are used as the immunizing agent, certain complications may occur. White* observed four instances of pneumonitis, one of endocarditis, and one of spontaneous pneumothorax in a group of horses in which living cultures had been eventually substituted after a course of immunization with heat-killed organisms. The pneumococci obtained from the lesions were apparently identical with the strains routinely injected. An interesting observation was that the serum of the horses at the time the infections occurred contained sufficient protective antibody so that 0.2 cubic centimeter of serum prevented the death of mice injected with 0.1 cubic centimeter of virulent culture, either of the organisms isolated from the horse or of those used in preparing the vaccine. These apparently paradoxical phenomena may be explained in part by the investigations of Griffith,[559] who reported that in rabbits the effect of intravenous inoculations of culture into an immune animal was to abolish temporarily the protective power of the serum. Griffith thought that the transient suppression of antibodies enabled the pneumococci to become established in the tissues of the animals. It seems, however, that any alleged superiority of serums from horses immunized with living cultures, unless the dosage is very carefully controlled, would be outweighed by the loss of animals from infections of the nature just described.

BLEEDINGS

Period of immunization prior to bleeding. The period of immunization necessary for the production in horses of sufficient protective antibodies to warrant initiating routine bleedings depends on the material injected, the spacing of doses, and the response of

* Unpublished observations.

the individual animal. Barach[75] studied the rate of development of immunity in mice and rabbits following administration of vaccine prepared from intact pneumococci of Types I and II and observed that protective substances developed three days after injection. The immunity increased markedly to the fifth day and remained essentially stationary to the seventh day. Barnes and White,[86] in studying the antibody response of rabbits to vaccines of Type I Pneumococcus, determined the average protective titer in a group of animals after twelve, twenty-four, thirty-six, and forty-eight injections of vaccine. The accompanying table illustrates the results obtained.

Course of injections	1	2	3	4
Number of injections prior to bleeding	12	24	36	48
Number of weeks between start of immunization and bleeding	6	12	24	36
Average protective titers	99	449	960	1500

It is probable that the protective potency of immune serum does not develop so rapidly in horses as in rabbits, and it has been believed that a period of from eight or nine months to a year or two of immunization might be required before a serum of satisfactory potency could be obtained. For over two years at the Massachusetts laboratory, as a result of studies on the immunizing efficacy of various antigens and injection schedules, all horses used in the production of antipneumococcic serum have been placed on routine bleedings approximately two months after the initiation of the immunization process. The plan has had no apparent harmful effect on the animals and, almost without exception, the serum has been found to be sufficiently high in protective antibody content to be suitable for concentration.

The interval to be allowed between the last injection of vaccine and the bleeding is somewhat elastic. By regulation of the National Institute of Health, at least one week must elapse in order that there may be no antigen circulating in the blood stream. Avery and his associates[36] stated that bleeding ordinarily took

place about ten days after the last injection of antigen. Barnes and White[86] observed that the highest titers in rabbits were usually found in seventh-day bleedings, occasionally in those on the twelfth day, and never when the blood was drawn on the twenty-second day. The authors therefore concluded that the optimal time for bleeding animals is in the period seven to ten days after the last injection of a series. According to the schedule adopted, the horses are bled on the ninth day after the last injection.

Amount of blood. The amount of blood which may be withdrawn from a horse depends on the size and physical condition of the animal. Usually from nine to twelve liters are taken.

Yield of serum. The yield of serum is subject to variation and is dependent in part upon the method used for collecting the blood, the number of bleedings to which the horse has been subjected and, apparently, to other more obscure factors. The yield obtained by using weights as an aid in expressing serum from the clot, as described by Avery and his associates, was from 50 to 55 per cent. If suitable equipment is available, the blood may be drawn directly into bottles of several liters capacity, and centrifuged after the clot has formed. In this manner the percentage of serum obtained may be slightly higher. When no special methods are employed and serum expressed by simple coagulation is drawn, the yield is likely to average only about 30 to 35 per cent of the total volume of blood.

There has been an impression in some laboratories that the amount of serum obtained from the first few bleedings was less than that from later bleedings. An analysis of results from a small group of horses under the observation of the authors of the present volume supports this belief. The data are taken from records of horses that had been bled from twenty-one to forty-six times. The average percentage yield was calculated for the first three, the middle three, and the last three bleedings, respectively, of each horse. Approximately 8 per cent more serum was obtained from the middle three bleedings as compared with the first series, and

about the same increase was noted in the last three bleedings when compared with the intermediate three.

The Production of Therapeutic Serum

REASONS FOR AND AGAINST THE USE OF UNCONCENTRATED SERUM

Until relatively recent times, the feasibility of concentrating and refining the specific serum used in the treatment of lobar pneumonia found scant recognition. Avery, Chickering, Cole, and Dochez[36] in 1917 believed that the disadvantages of the concentrated product were such as to render it of little practical value. In a later publication, Cole (1929),[265] although recognizing that treatment of pneumonia with large doses of whole serum was not ideal, questioned seriously the advisability of using concentrated preparations. Cole objected to the methods used for standardization and doubted whether the small doses of concentrated antibody recommended would have any effect on the mortality rate. He concluded that unless very large doses of concentrated serum were employed or unless more accurate methods of standardization were adopted, it would be wiser to continue to treat cases of Type I pneumonia with potent, whole serum in large doses.

Sickles (1929)[1278] offered a comparison of the protective titers of unconcentrated and concentrated serums. The results led to the impression that unconcentrated serums were superior to concentrated products because of zonal phenomena in the action of the concentrates, and because, in her experience, the potencies of the two kinds of preparations were not essentially different. Much of the objection to concentrated serum voiced by Sickles was based on unsatisfactory results attending the methods of standardization employed. In 1932, Wadsworth,[1463] in discussing the question, stated that there were no clinical data to indicate that refined products were more effective in the treatment of pneumonia than whole serum of the highest potency and, furthermore, that before

introducing new methods of preparation it was of importance to be certain that during the process of concentration the serum would lose no activity that might be effective in treating the disease. Wadsworth also maintained that the existing methods of standardization were inadequate, although it was acknowledged that the potency of concentrated serum approximated that of the unconcentrated product. It is difficult to concede the cogency of an argument in favor of unconcentrated serums based on difficulties in estimating the content of antipneumococcic protective antibody, since the methods for determining potency are alike for both whole and refined products.

Park,[1053] in the Harben Lectures, discussed the entire question and stressed some important points which may be included here. Park stated that in cases of pneumonia it is desirable to avoid unnecessary administration of serum, but when the use of serum is indicated, its disadvantages should be borne in mind. Large intravenous doses of unrefined serum have frequently resulted in attacks of serum sickness, and the technique of administration presents difficulties in the hands of those inexperienced with the method. By concentration methods, serum of lower potency can be utilized and a satisfactory product obtained, thus resulting in reduced cost. Five cubic centimeters of concentrate containing two thousand protective units per cubic centimeter should equal the protective value of one hundred cubic centimeters of serum containing one hundred protective units per cubic centimeter. The dose of the former, containing less foreign protein, being smaller in quantity, and easier to administer, would be superior to whole serum. Park concluded, therefore, that the refining of antipneumococcic serum provides a preparation preferable to whole serum. The Therapeutic Trials Committee of the Medical Research Council in England,[887] in a report on the serum treatment of lobar pneumonia, stated that delayed reactions were rare and slight with concentrated serum, whereas with unconcentrated serum of high titer the treatment more frequently caused rashes, swollen joints, and

pyrexia. Unconcentrated serum, however, was quite as effective as concentrated serum if the same number of units were used. That the concentrated product is effective therapeutically has been amply demonstrated, and confirmatory data have been summarized by Lord and Heffron (1936).[827]

AVIAN SERUM

The therapeutic use of antipneumococcic serum produced by repeated injections of Brahma cockerels with virulent pneumococci has been reported by Kyes (1918).[767] Severe constitutional reactions followed the administration of the serum to human beings. Although later publications by Kyes and Carey (1927, 1929)[768-9] described methods for eliminating the factors believed to be responsible for the reactions, fowl serum has not been generally accepted for the treatment of lobar pneumonia. The small size of the birds used, resulting in many technical disadvantages, is an objection which, in the opinion of the authors of this volume, would discourage its routine use.

POLYVALENT SERUM

Prior to the separation of pneumococci into specific serological types, it was customary to prepare antiserums against individual strains. The serums were tested serologically against cultures from heterologous sources. Neufeld and Haendel[991] found that some of the earlier, so-called polyvalent serums reacted with only one type of Pneumococcus. Cole[249] reported that a univalent serum used by himself and colleagues protected animals against only 40 per cent of the cultures tested. Cole and Moore[268] emphasized the importance of using pneumococci of strict immunological specificity for immunization and, inasmuch as at the time the only serum of proved therapeutic value was for Type I, monovalent serum was recommended. In the same year, 1917, Avery and his associates advised against the routine manufacture of polyvalent antipneumococcic serums.

Subsequent to the division of pneumococci into types by Dochez and Gillespie,[322] Cooper, Edwards, and Rosenstein,[272] in separating Group IV pneumococci into twenty-nine types, found little cross-protection of monovalent serums against heterologous strains. For example, a concentrated Type II serum contained only ten to twenty units against a Type V culture. In 1932, however, Cooper, Rosenstein, Walter, and Peizer[274] suggested that, because of the marked cross-reactions of many Type V strains with Type II antiserums and *vice versa,* it might be advisable to prepare an antiserum potent for both types. Observations by Barnes and Wight (1936)[88] which suggested that, under stated conditions, Type V Pneumococcus might become transformed into Type II Pneumococcus, led them to make a recommendation in accord with that of Cooper and her colleagues.

Aside from such a basis for the manufacture of bivalent or polyvalent serums, there are other factors which should be taken into consideration. In some laboratories, serum bivalent for Types I and II has been produced. Since there has been no demonstration of close relationship between the types, the only justification for the custom appears to be one of economy. This explanation also holds true in the case of serums polyvalent for other pneumococcal types. Horses appear to tolerate injections of a vaccine containing pneumococci of several types as well as of an antigen made from a single strain. Antibodies active against the various strains used can thus be produced in one animal instead of using separate horses for each type serum desired. Whether the economy is real or apparent is a question which remains for the producer to determine. On the other hand, an impression, confirmed, at least in part, by Cooper and her associates,[272] has been gained, that higher protective powers are developed in monovalent immunization than is the case when strains of several types are injected into producing animals. Definite evidence to support the hypothesis is, however, lacking. With the rapid and accurate Neufeld method of typing

sputum now in general use, the former practice of administering a dose of bivalent or polyvalent serum during the period required for type diagnosis is no longer necessary. In view of present-day knowledge, a logical and conservative procedure would be the production of monovalent Type I serum, bivalent Type II and V, possibly a serum combining Type III and VIII antibody, and either bivalent or polyvalent serums for other closely related types.

METHODS OF CONCENTRATING SERUM

The objectives of the processes employed in the concentration and refinement of antipneumococcic serum are to eliminate non-essential and harmful constituents and to reduce the volume of fluid while conserving specific protective substances. In a study of the distribution of immune bodies occurring in antipneumococcic serum, Avery,[32] by chemical methods, demonstrated that the antibodies in antipneumococcic serum are associated with the globulins and not with the albumin fraction. Gay and Chickering (1915),[508-9] and later Chickering (1915),[223] showed that the precipitate which forms when an extract of pneumococcal cells is mixed with homologous antiserum contains practically all the immune substances and that the antibodies can be recovered from the precipitate in a water-clear solution by treatment with dilute alkali. By a similar method, Huntoon *et al.* (1921)[665-6, 668] precipitated the specific antibody from antipneumococcic serum by treatment with heavy suspensions of pneumococci. Various procedures have since been utilized for the purpose of dissociating the antigen-antibody complex so formed and of preparing for therapeutic use solutions of the specific antibodies so released.

Biological methods. The biological methods of concentration employed by Gay and Chickering and by Huntoon are of interest in establishing immunological principles. Although, as stated in the Rockefeller Monograph, the products thus obtained were ideal in many ways, there were serious disadvantages in the method. The

technical manipulations were laborious, the preparations varied in strength, were not stable, and frequently gave rise to severe reactions in patients.

Chemical methods. Chemical methods of refining antipneumococcic serum are more practicable and have received greater recognition.

Ammonium Sulfate: In the investigations made by Avery,[32] the serum used was obtained from horses immunized with cultures of pneumococci of Types I and II. Avery's work showed that the antibodies in antipneumococcic serum are absent from the albumin fraction but associated with the globulins, and while not associated wholly with pseudoglobulin, precipitation by 38 to 42 per cent saturation with ammonium sulfate offered a promising method for the practical purification of antipneumococcic serum.

Avery's experiments with ammonium sulfate were confirmed by the investigations of Banzhaf (1925),[70] which stimulated a continued interest in the method. By a modification of Avery's method, and using plasma instead of serum, Brown and Knowles (1932)[153] attempted to increase the yield of antibody and to determine the comparative protective value of the euglobulin and pseudoglobulin fractions. The percentage recovery obtained is not clear, but the results suggested that the bulk of protective antibody is to be found in the pseudoglobulin.

In studying the "non-antibacterial" factors in the therapeutic action of immune serum on dermal pneumococcal infection in rabbits, Sabin (1933)[1206] used the ammonium sulfate method of fractionation. A zonal effect was observed in the experiments, and, because of this, Sabin stated that "the 30 to 50% ammonium sulfate globulin, either total or water-insoluble, would be quite undesirable for therapeutic purposes." Felton and Kauffmann (1933),[429] on the other hand, found that the highest protective potency for mice in relation to the amount of protein was in portions precipitated by ammonium sulfate between 36 and 50 per cent saturation, thus corroborating Avery's observations.

Several different lots of antipneumococcic serum, bivalent for Types I and II, have been concentrated by ammonium sulfate at the Massachusetts Antitoxin and Vaccine Laboratory. The percentage recovery in all cases has been high, averaging approximately 90 per cent of the original antibody and, furthermore, the products have possessed the desirable physical characters. Chill-reactions have followed the administration of some of these lots, however, in a fairly high percentage of cases. Although it has been possible by reconcentration processes to render batches relatively free from this undesirable property, it is not definitely clear that the method was at fault.

Sodium Sulfate: In Banzhaf's[70] experiments it was found that solutions of precipitate obtained from antipneumococcic serum by adding 12.5 per cent dried sodium sulfate contained after dialysis only 8 to 10 per cent of the total immune bodies. If, however, a filtrate of globulins, soluble in 12.5 per cent sodium sulfate, was treated with sodium sulfate up to 18.5 per cent at a temperature of 36°, the preparation after dialysis contained about 90 per cent of the total antibody. Felton (1928)[403] reported detailed observations on the concentration of serum by the use of sodium sulfate, which method gave a product suitable for intravenous therapy. In a later publication by the same author (1931),[403] some disadvantages of the antibody solution prepared by the sodium sulfate method were acknowledged.

Banzhaf and Klein[72] made a further report on the use of sodium sulfate in a normality of about 0.05 for precipitating dialyzed antiserum. In the process, the reaction was corrected to pH 5.0 to 5.1 and the precaution was believed to result in a chill-free product. Savino, Negrete, and Acuna,[1221] using plasma instead of serum, applied a sodium sulfate method of concentration substantially the same as that of Felton. Although the method undoubtedly marked an advance in refinement procedures, it was somewhat expensive, and difficulties attended its execution particularly in the relative insolubility of the antibody at different stages of the re-

fining process, in filtration for sterilization, and in obtaining lots consistently free from chill-producing elements.

Dialysis: The removal of crystalloids by the dialysis of serum in water was studied by Avery[32] as a means of concentrating antipneumococcic serum. The procedure resulted in an incomplete precipitation of antibody. Dialysis, however, may be utilized to advantage at some stage in processing serum by salting-out methods. Banzhaf[70] found that serum precipitated by sodium chloride or ammonium sulfate contained about 90 per cent of the immune bodies following dialysis. Later Banzhaf and Klein[72] recommended dialyzing serum until practically free from salts as the initial step in eliminating inert protein and chill-producing substances. Goodner[528] reported that it was unnecessary in dialysis to remove electrolytes completely in order to obtain a precipitate containing the majority of the agglutinins. The method of dialysis has certain advantages not possessed by other processes of concentration. It is inexpensive and frequently results in a volume concentration not readily obtained by other procedures, and it entails less harsh physical treatment of the antibody than the addition of chemicals. Some unpublished data, however, show that solutions of antibody made by the method are not stable.

Dilution with Water: Felton (1924)[396] observed that a heavy precipitate formed when antipneumococcic serum was diluted in a proportion of 1 to 10 with water, and believed that the amount of water-insoluble precipitate so obtained gave an indication of the protective strength of the serum. A higher yield of precipitate was recovered when small amounts of tartaric, citric, oxalic, or acetic acids were added to the water. Experiments on the influence of hydrogen ion concentration on the precipitation of immune substances from specific serum suggested that the isoelectric zone of the protective antibody lies between pH 6.6 and 7.5, possibly at about pH 6.8.

In later papers, Felton[400] and Felton and Bailey[421] reported additional experiments dealing with the water precipitation of pneu-

mococcal antibody. The amount of precipitate obtained did not always parallel the protective titer of the antibody solutions. The optimal yield of precipitate varied with dilution and the serological type of the serum used. Thus dilutions of 1 to 18 and 1 to 20 for Type I, 1 to 14 and 1 to 18 for Type II, and 1 to 12 and 1 to 14 for Type III serum were recommended as yielding maxima of antibody. The amounts thrown down were, however, also dependent upon the hydrogen ion concentration of the water-serum mixture, and zones of pH 5.5 to 7.8, 5.5 to 7.8, and 6.2 to 7.8 were observed for serum of Types I, II, and III respectively.

Banzhaf[70] corroborated Felton's observations on Type I serum diluted tenfold with water containing phosphoric acid, and obtained varying results with Type II and III serum similarly treated. In 1928, Felton[403] published a further description of the characters of the water-insoluble fraction of antipneumococcic serum, and believed that the fraction, while similar in qualities to pseudoglobulin, more closely resembled euglobulin.

Felton's work received further confirmation in the experiments of Savino, Negrete, and Acuna.[1221] Reiner and Reiner,[1133] using a similar method of concentration, found that globulin can be precipitated from both normal and antipneumococcic serum by dilution with distilled water at hydrogen ion concentrations varying from pH 5.0 to 7.0. The zone of maximal precipitation was observed to lie between pH 5.5 and 6.0. By repeated precipitation at pH 5.1 and pH 6.8, respectively, two fractions were obtained, one of which was more soluble at neutral, the other at acid reaction. It was shown that the two fractions combine with each other if the solutions are mixed. The latter observation is of significance in connection with the concentration of antipneumococcic serum bivalent for Types I and II.

After experimenting with water precipitation, Goodner[528] recommended the employment of preliminary water tests for each lot of serum to determine its suitability for concentration. By observing the dilution of serum with distilled water necessary to cause

opalescence—the initial point—it was possible to calculate the total amount of water necessary to obtain maximal precipitation. One of the requirements of the procedure was the maintenance of low temperatures with the materials used. Banzhaf and Klein[72] employed a somewhat similar process except that adjustments in hydrogen ion concentration were made, but the authors stated that Goodner's method probably yielded a purer form of antibody despite the presence of chill-producing agents.

There is some evidence that the percentage recovery of Type II antibody is appreciably lower than that of Type I. It has been suggested that the disparity might be due to a difference in the isoelectric points, or zones, of the two types of antibody, and that Type II immune substances might precipitate more completely at a lower hydrogen ion concentration without affecting the yield of Type I immune substances. The question of the solubility of one antibody fraction in the other has also been raised by Savino, Negrete, and Acuna. Observations by Hager, Barnes, and Wight* (1935), are pertinent to the subject. From a single, pooled lot of antipneumococcic serum of Types I and II, four different batches were concentrated by the alcohol method and the antibody precipitated at pH 5.0, 6.0, 7.0, and 8.0, respectively. Repeated mouse tests gave the following approximate percentage yield of protective substances: At pH 5.0, Type I, 83 per cent and Type II, 66 per cent; at pH 6.0, Type I, 80 per cent and Type II, 59 per cent; at pH 7.0, Type I, 98 per cent and Type II, 93 per cent; at pH 8.0, Type I, 98 per cent and Type II, 85 per cent. No acid fraction was removed from the solutions. The results suggest that, under the experimental conditions employed, antibodies of both Types I and II are precipitated more completely at pH values of 7.0 and 8.0 than at 5.0 and 6.0, with the optimum at neutrality or thereabouts. The results are also in essential agreement with the earlier observations made by Felton, and by Felton and Bailey, regarding the isoelectric points of antipneumococcal antibodies. It is possible

* 1935, unpublished.

that concentration of antipneumococcic serum by water precipitation might be developed into a practical method. Water subjects the antibody to as little physical injury, perhaps, as any of the processes employed. If modifications were made so that a high yield of antibody free from reactive substances could be obtained, the method would also possess the added advantage of being highly economical.

Carbon Dioxide: Avery reported that after diluting antipneumococcic serum with one-half its volume of water saturation with carbon dioxide resulted in an incomplete precipitation of the antibodies. Felton[396] repeated the experiment and as Avery had found, noted that the yield in protective substance was never more, and usually less than one-half the amount contained in the original serum. The method of concentration with carbon dioxide has little value from the practical standpoint.

Sodium Chloride: Avery found that saturation of serum with sodium chloride, as with carbon dioxide, resulted in a low yield of antibody, since the immune substances were apparently associated with both the euglobulin and pseudoglobulin separated by this treatment. Banzhaf (1925)[70] corroborated the observation and determined that the euglobulin so obtained contained only 8 to 10 per cent of the immune bodies.

Alcohol Precipitation: From the sodium sulfate method, Felton (1931)[408] turned to the use of ethyl alcohol as a precipitant in concentrating antipneumococcic serum. Briefly, the technique recommended is as follows: Ethyl alcohol (95 per cent) and the serum are cooled to 0°, and the alcohol is added to the serum in the proportion of 20 cubic centimeters of alcohol to 100 cubic centimeters of serum. The mixture is allowed to stand for eighteen hours in the cold, and the precipitate which forms is then recovered either by centrifugation or filtration. The precipitate is washed with one and one-half volumes of cold distilled water to remove the water-soluble protein. The insoluble fraction is dissolved to the volume desired in isotonic salt solution. The so-called acid

fraction may be removed by suspending the alcoholic precipitate in water or by adding sodium chloride to effect solution, then adjusting the reaction to pH 5.0. The precipitate thus formed is separated by centrifugation. The importance of maintaining an even, low temperature during contact of the alcohol with serum is emphasized because a slight increase in temperature was believed to result in solution of the precipitate and in partial destruction of antibody. A yield of at least 80 per cent of the protective substance originally present in the serum is obtained by this method.

Felton reported that several different lots of serum concentrated by the method had been used therapeutically without causing untoward reactions. Some modifications of the original technique have been found advisable during routine practice, chiefly those directed toward avoiding denaturation of the immune globulin.

Other Methods: An objectionable feature of the antibody solutions prepared by the methods of Gay and Chickering and of Huntoon is the difficulty in separating the cellular components of Pneumococcus from the antigen-antibody precipitate. Felton and Bailey[420] found that 0.01 molar sodium carbonate was the most satisfactory of the agents tried for dissociating the polysaccharide-antibody combination. Dissociation took place on heating the specific precipitate in the presence of sodium carbonate, disodium phosphate, saccharose, or sodium chloride, and the protective substance could then be precipitated by sodium sulfate. The released antibody, however, was found to contain a substance antagonistic to specific protection, thus suggesting that not all the capsular polysaccharide had been removed.

In a later study, Felton[410] reported that calcium or strontium phosphate could also be used to dissociate specific carbohydrate-antibody precipitate yielding a water-insoluble protein that conferred protection on mice. Felton and Kauffmann[429] observed that the zone of minimal solubility of the immune fraction isolated by

calcium phosphate dissociation of an SSS-antibody complex was pH 6.7 and above, corresponding roughly to the fraction of immune serum obtained by 36 to 50 per cent saturation with ammonium sulfate. The various modifications of the methods of Gay and Chickering and of Huntoon are of academic interest and afford means of studying and analyzing the immune substances, but it seems unlikely that the procedures have sufficient practical application to warrant consideration for large-scale concentration of serums. The probable presence of residual antigenic substance in preparations made by either the original or modified methods is one of the more important objections to the antibody solution obtained in this manner.

The use of metallic salts in preparing antibody solutions was introduced by Felton[410] during studies designed to increase the yield of immune substances obtained by alcohol precipitation. Treatment of the total water-insoluble protein of antipneumococcic serum with either aluminum chloride at pH 5.2 or with zinc chloride at pH 7.0 caused heavy precipitation, with the immunologically active protein remaining in solution as the metallic salt. When freed from zinc by means of a carbonate or phosphate, the water-insoluble protein was highly protective for mice. As far as can be learned, antibody solutions prepared by this or similar methods have not progressed beyond the experimental stage.

The use of ultrafiltration as a means of purifying antibody is a more recent development. Goodner, Horsfall, and Bauer (1936)[539] applied the method to rabbit serum, horse serum, and concentrated horse serum containing antibody for Type I pneumococci. With membranes having a pore diameter of 102.5 millimicrons, the filtrates of the horse and rabbit serums contained 76.7 and 86.6 per cent respectively of the total antibody, while at a pore diameter of 188 millimicrons the filtrate of concentrated horse serum contained 100 per cent of the antibody. It is possible that ultrafiltration may prove to be of practical value in the routine production of concentrated antipneumococcic serum.

The recent description by Wyckoff[1555] of ultracentrifugation as a means of concentrating antipneumococcic serum is of interest. By use of a vacuum centrifuge having a maximal field of about forty thousand times gravity it was possible to separate the serum constituents into different layers. The sedimented portion was found to contain only those protein molecules that were considered to be antibody. These observations, if confirmed, promise to be of practical value in the production of antipneumococcic serum.

Of the methods of concentrating antipneumococcic serum discussed in this section, the one employing alcohol as a precipitant is at the present time in more general use than other processes. The recovery of antibody is fairly satisfactory, the method is economical, the end product usually possesses the desired physical characters, and results following its intravenous administration are generally favorable and do not include an undue number of disagreeable reactions.

PHYSICAL PROPERTIES

Total solids. The physical characters of the product are of importance because of the fact that antipneumococcic serum is administered intravenously. By regulation of the National Institute of Health, serums intended for use in intravenous therapy should not be of excessive viscosity or have a content of total solids greater than 20 per cent. Unconcentrated serums usually contain about 8 or 9 per cent of solids, whereas the amount present in concentrated antibody preparations varies with the method of processing and with the degree of concentration attained. An ideal product in this respect would be one containing a high antibody content with the viscosity and total solids at points permitting ease in handling. Hence, from a practical standpoint it is well to maintain the total solids content of concentrated antipneumococcic serum at about 10 to 12 per cent.

Inorganic solids. Determination of the ash in antipneumococcic serum is unnecessary as a routine procedure, but is of value as a

check on the amount of salt added. No concentration of salt higher than that sufficient to effect complete solution of the antibody precipitate should be used. For the majority of preparations 0.85 per cent suffices; concentrations higher than one per cent should not be required.

Reaction of the product. The buffering action of the blood is ordinarily considered sufficient to compensate for minor fluctuations in reaction from neutrality of substances introduced intravenously, but a system already strained by disease should not be required to make any unnecessary adjustment of hydrogen ion concentration in the blood. Antipneumococcic serum and concentrates used for clinical purposes, therefore, should be approximately neutral in reaction. The usual preparations for therapeutic use show hydrogen ion concentrations ranging from pH 6.0 to 7.0, with the average approaching pH 6.5, and hence require no readjustment.

Preservatives. There is no substitute for sterility in products intended for therapeutic use. It is customary, and is required by governmental agencies, to add a preservative to antipneumococcic serum. In some laboratories, the antiseptic is added when the serum is drawn from the clot. The precipitate which sometimes forms, sediments during storage in the cold. Other manufacturers prefer to add the preservative at some stage during concentration or final processing. In either case, the preservative should be added prior to final filtration. The purpose of the preservative is not to destroy contaminating organisms but to prevent the growth of any adventitious bacteria.

The choice of preservative, and the concentration of the bactericidal agent, are questions of no small moment. Investigations conducted by Hale (1913)[586] with tricresol when used as a preservative in antimeningococcic serum led him to conclude that the agent is dangerous when serum is to be introduced by a route that brings the agent into direct contact with the nervous centers. Leake and Corbitt (1917)[794] attempted to determine the tox-

icity of phenol, cresols, and glycerol by physiological tests on
mice. It was impossible to establish a definite minimal lethal dose
of the substances because of marked variations in susceptibility of
the test animals. It was found, however, that tricresol and phenol
exerted approximately the same germicidal effect. Voegtlein
(1918)[1451] studied the effect of the addition of tricresol and phenol
to antipneumococcic serum introduced intravenously into mon-
keys, cats, and dogs. The doses given were calculated to corre-
spond to the amounts given in the treatment of pneumonia. Voegt-
lein concluded that the addition of tricresol or phenol to antipneu-
mococcic serum in concentrations not exceeding 0.5 per cent does
not seem to impart to the serum toxic properties which might give
rise to serious symptoms following its intravenous administration.
It was suggested, however, that serum containing phenol should
not be given to patients with nephritic complications because of
the injurious action of phenol on the kidney.

A mercurial preparation, Merthiolate, has been recommended
as a preservative for biological products. Jamieson and Powell
(1931)[677] reported that the substance is highly effective as a
germicide, possesses low toxicity, and is not subject to precipita-
tion in protein solutions. The agent has not, however, come into
general use as a preservative in antipneumococcic serum.

Malcolm (1931)[857] studied the germicidal and toxic effects of
various chemical substances, and found that the mercury com-
pounds, Metaphen and Mercurophen, possessed higher bactericidal
powers than phenol and tricresol, and were relatively less toxic,
thus permitting a wider margin of safety when used as preserva-
tives for biological products. Another advantage possessed by the
two mercury compounds is that at their effective germicidal
strength they do not precipitate proteins. Of the preservatives
studied, Malcolm concluded that Mercurophen was the most satis-
factory, with Metaphen second in order of effectiveness, and that
both were superior to phenol or tricresol. At the present time both
phenol and tricresol in final concentrations not exceeding 0.5 per

cent are in general use. At the Massachusetts laboratory, 0.2 per cent phenol has been found to be satisfactory in maintaining the sterility of therapeutic antipneumococcic serum. Care must be taken that precipitation of protein does not follow the addition of phenol and tricresol.

Interest in the drying of serum as a means of preservation has recently been revived, largely through the investigations of Mudd, Reichel, Flosdorf and Eagle,[934] Elser, Thomas and Steffen,[354] and Flosdorf and Mudd (1935).[454] The methods employed are applications of familiar physical conditions, namely, the use of low temperatures and absence of moisture and oxygen. A detailed description of the equipment used for desiccating biological products, now known as the "Lyophile apparatus" may be found in the publication of Flosdorf and Mudd.[454]

CHILL-PRODUCING FACTORS

A disturbing, and as yet unsolved, problem is the occasional occurrence of a thermal reaction, or chills, in patients following the administration of some lots of antipneumococcic serum. For a discussion of the clinical manifestations and management of the reaction the reader is referred to *Lobar Pneumonia and Serum Therapy* by Lord and Heffron[827] and the more comprehensive treatise by Heffron.[601] The incidence of chills varies with different lots of serum and with the method of refinement in the case of concentrated products.

During the development of various methods of concentrating antipneumococcic serum, attempts have been made to eliminate non-essential constituents for the purpose of reducing reaction-producing propensities. Unfortunately, some of the manipulations involved are likely to result in varying degrees of denaturation of the proteins associated with the protective antibody. Denaturation may be considered as such an alteration in serum proteins that they become insoluble in solutions of neutral salts. That these changes may occur readily is shown by the work of Wu and Yen

(1924).[1553] Felton[398] observed that, in general, agents that cause denaturation of the globulins in antipneumococcic serum also result in a destruction of protective antibody. The addition of acids, alkalies, and preservatives—particularly chloroform, which coagulates the water-insoluble globulin—repeated solution in hydrochloric acid and precipitation by sodium hydroxide, or frequent solution in sodium chloride and precipitation by water at room temperature were all observed by Felton to result in a modification of the immune globulin.

Felton[403] also stated that serums which were stored for a long period of time, with or without preservative, undergo denaturation and, in describing the sodium sulfate method of concentration, listed three common causes which may be controlled, namely, bacterial contamination, maintenance of serum or precipitate at too high a temperature in acid or alkaline solution, and too high a concentration of preservative. Excessive heat also alters the solubility of the protective globulin in antipneumococcic serum (Felton[397]). Impurities and the hydrogen ion concentration of agents used as precipitants in the concentration process may also contribute to the change in the protein. In the report on the use of sodium sulfate, Felton emphasized the point that the salt may be markedly alkaline and require neutralization prior to use. Serum concentrated by ammonium sulfate, although satisfactory in many ways, frequently elicits a high incidence of thermal reactions, possibly due in part to changes in the serum protein. Observations by Cianciarulo (1935)* on the hydrogen ion concentration of ammonium sulfate offer a possible explanation for alterations in protein antibody when purified by means of the ammonium salt. The reaction of some lots of the sulfate was found to be as acid as pH 4.4 and, when used in concentrating diphtheria antitoxin, resulted in marked denaturation as evidenced by the formation of an insoluble, jelly-like precipitate. The salt, with the reaction properly adjusted, no longer caused this effect.

* Unpublished.

Aside from the resultant loss of antibody and difficulty in effecting solution occasioned by denaturation of globulins, there is the possibility that the chemical or physical alteration may be responsible for the chills following therapeutic administration of concentrated serums.

Studies designed to determine the cause of chill-reactions and methods for eliminating chill-producing principles in antipneumococcic serum have followed several directions. The reactions are not confined to the use of antipneumococcic serum, according to Rhoades.[1135] Larson and Fahr (1925)[790] observed marked chills in three of seven patients treated with antipneumococcic rabbit serum. Differences between immune horse and rabbit serums have been studied by Goodner and Horsfall (1935),[537] and by Goodner, Horsfall and Bauer (1936),[539] while Chow (1936)[224] recently reported that the major portion of antibody in antipneumococcic rabbit serum occurs in a different globulin fraction from that in horse serum. Whether these differences may explain in part the high incidence of chills following therapeutic use of untreated rabbit serums remains unknown.

A different approach to the problem was reported by Kolmer and Matsumoto (1920),[744] who studied normal and immune horse serum for the content of hemagglutinins and hemolysins to ascertain whether intravascular agglutination or hemolysis might play a part in chill reactions incident to serum therapy in pneumonia. Although it was found that practically all horse serum contains agglutinins for human erythrocytes, the authors believed that the intravenous administration of horse serum probably does not introduce sufficient amounts of hemagglutinin and hemolysin to produce ill effects referable to agglutination and hemolysis. Bullowa, Rosenbluth, and Merkin (1927),[184] as a result of an incidental observation that a chill-producing serum agglutinated red cells *in vitro* whereas a chill-free serum did not, restudied this phase of the question. No constant relation was found between erythrocyte agglutination *in vitro* and serum reactions in man, thus confirming

the earlier impression of Kolmer and Matsumoto. In spite of these concurring opinions, the hypothesis should be subjected to further investigation, including a study of the effect of heterophile antibodies. Sabin and Wallace (1931)[1210] reported the results of studies on the nature of the chill-producing principle in antipneumococcic serum. They were unable to find any relation between the reaction and the presence of formed elements, fibrinogen, or lipids, but confirmed Felton's belief that the agent in the acid fraction is probably carried by the globulin through an adsorptive process.

The administration of antipneumococcic fowl serum to pneumonia patients was found by Kyes (1918)[767] to result almost invariably in severe chills. In a later report, Kyes and Carey (1927)[768] suggested that the antecedents of fibrin present in serum, by favoring the formation of fibrinous emboli in the blood stream, were responsible for serum shock. By treating the serum with calcium or cephalin and removing the resultant precipitate, the authors claimed to have rendered fowl serum free of shock-producing qualities. Meyers (1932)[900] reported a series of studies in which titrations were made of the fibrinogen content of antipneumococcic horse serum. The investigations appear to be chiefly concerned with serum disease rather than with chill-reactions, but from the results the author concluded that fibrinogen was not involved. The intravenous injection of reasonably pure fibrinogen failed to cause reactions, whereas concentrated serum, containing no detectable fibrinogen, not infrequently produced serum shock.

The acid fraction. In 1928, Felton,[403] after describing his method of concentrating antipneumococcic serum by the use of sodium sulfate, recommended the removal of a precipitate that formed when the reaction of the dialyzed material was adjusted to pH 4.6 to 4.8. The supernatant fluid, after removal of the acid fraction, was readjusted to pH 6.8 and diluted with distilled water. The white precipitate thus formed, when dissolved in salt solution, was reported to be practically free from severe chill-producing properties. In studying the possible causes of chill-pro-

duction, Felton (1928) found that removal of the acid fraction from the crude, water-insoluble precipitate carried most of the phosphorus and lipids, but the author hesitated to conclude that these substances were entirely responsible for chill-production, although concentrates so prepared were reported to be essentially free from the substance provoking reactions. In 1930, Felton[404] stated that, notwithstanding the fact that elimination of the acid fraction was the most important single step in preparing chill-free concentrates, toxins due to bacterial contamination during manufacture and storage might also be a contributory factor. In comparing chill-producing concentrates with bland preparations, Felton and Kauffmann (1931)[427] found the former to contain some residual acid fraction, a higher phosphorus and lipid content, and more ammonia and non-protein nitrogen. Of these factors, the acid fraction was considered to be the most important. However, it is not always possible to obtain an acid fraction precipitate from chill-producing lots as reported by Barnes and Kramer (1933).[83] Complete reconcentration of the serum may render some, but not all, of the preparations suitable for use (Park, 1930[1053]).

Banzhaf and Klein (1930)[72] described the details of a method of preparing chill-free concentrates. After dialysis, sodium sulfate or sodium chloride is added and, after storage in the cold overnight, the reaction is adjusted to pH 5.1 to 5.3. Insoluble substances, fibrin, fibrinogen, euglobulin, and chill-producing substances flocculate and are removed. The material is filtered and the reaction of the fluid is adjusted to pH 6.8 for further precipitation, principally of fibrin. The sediment is removed by filtration and the water-insoluble precipitate prepared by the usual technique. In the alcohol method of concentration, Felton (1931)[408] advised the removal of the acid fraction at pH 5.0. Concentrates prepared by this means, particularly when precautions are taken to avoid denaturation by the alcohol, have been for the most part satisfactory, although lots that occasion reactions are sometimes encountered.

Tests for chill-producing properties. Until the nature of the chill-producing factor is known and until the substance can be removed from antipneumococcic serum, it is necessary, prior to distribution of the product, to have recourse to some means of determining the presence or absence of this property. It is not expedient to make preliminary tests on human subjects, and the smaller, more commonly used laboratory animals are unsatisfactory for the purpose. Sabin and Wallace[1209] stated that it was possible to differentiate chill-producing from satisfactory lots of serum by intravenous injection into suitable dogs. A rise in temperature of 1.5°F. or more was considered the criterion of a positive reaction, regardless of the presence or absence of a definite chill. In a later and more complete paper, Sabin and Wallace[1210] reported observations on a considerable number of serums tested by the method, and confirmed their previous results. Barnes and Kramer (1933)[83] made a preliminary report on the use of monkeys (*Macacus rhesus*) in detecting chill-producing lots of concentrated serum and found the response of the animals to correspond closely with those observed in patients with lobar pneumonia treated with the preparations. In the test animal, the presence or absence of frank chills appeared to be of more significance than elevation of body temperature.

In comparing the products obtained by the sodium sulfate and alcohol methods, Barnes and Kramer[83] tested a pooled lot of antipneumococcic serum that had been divided into two equal parts. One batch had been concentrated by sodium sulfate precipitation, the other by alcohol. The sodium sulfate concentrate gave chills in a high percentage of cases, while the batch refined with alcohol was essentially chill-free in its action.

The practical value of the method was affirmed by the observations of Barnes and Robinson (1936),[84] who found that application of the test over an extended period of time and the rejection of lots of serum eliciting chills in the test monkeys had reduced the

incidence of chills by at least 50 per cent in patients undergoing antipneumococcic serum therapy.

Under existing circumstances, it is therefore advisable to utilize some method for selecting only chill-free concentrates for clinical use. Lots found by preliminary tests to possess this unwanted property may be reprocessed, with particular attention paid to solubilities at various hydrogen ion concentrations.

Potency Tests

In the manufacturing laboratory, the determination of the antibody content of antipneumococcic serum is of economic importance in measuring the response of animals during the process of immunization and in selecting serum suitable for therapeutic use. In the clinic it is essential to know the potency of the preparation being used in order to obtain more uniform dosage and to estimate its effectiveness. Standardization of antipneumococcic serum involves difficulties not encountered in the assaying of such biological products as diphtheria and tetanus antitoxins. Progress has been made, however, and, as Park and Cooper[1057-a] observed, although the unit of pneumococcal antibody cannot be so accurately estimated as the diphtheria antitoxic unit, the present standard suffices for therapeutic purposes.

IN VIVO TESTS

During the development of methods for standardizing antipneumococcic serum, both *in vivo* and *in vitro* tests have been utilized. The former have included procedures for determining the protective and the therapeutic qualities of serum, and the latter have been concerned with estimating the content of antibodies demonstrable by serological reactions.

Early animal tests. Washbourn[1487] injected intraperitoneally into rabbits mixtures of measured amounts of serum and a quantity of culture representing ten times the minimal fatal dose. A

unit of antibody was defined as the smallest amount of serum that
would protect rabbits under the conditions of the test. High viru-
lence of the cultures for rabbits was maintained by special meth-
ods. Washbourn also tested the therapeutic action of serum in rab-
bits and gauged the dosage on the basis of units of antibody. Eyre
and Washbourn[374] reported further experiments on standardiza-
tion, employing mice and administering the mixtures subcutane-
ously. The authors stated that there was no relation between pro-
tection, agglutination, and bactericidal action of the serums—an
observation that must be discounted in view of evidence obtained
by more recent investigators. In a later publication, Eyre and
Washbourn[377] announced the method adopted by them for stand-
ardizing antipneumococcic serum, which depended upon determin-
ing the protective power of 1.0 cubic centimeter of serum in terms
of the number of fatal doses of highly virulent pneumococci in-
jected. The serum was administered intravenously to rabbits and
was followed immediately by an intraperitoneal inoculation of cul-
ture, the minimal lethal dose of which was determined by injecting
various dilutions of the culture into control rabbits.

In testing the protective value of various serums, Römer
(1902)[1155] made simultaneous injections of culture and of serum,
using both mice and rabbits and, in 1905,[1156] he made a further
report of his technique in which a series of mice were inoculated
subcutaneously with decreasing doses of serum. After twelve to
seventeen hours the test animals and the controls were injected
with 10 to 100 lethal doses of culture. For the purpose of inter-
preting results, Römer termed a serum of which 0.01 cubic centi-
meter protected animals a "simple serum" and assumed that one
cubic centimeter of simple serum constituted one "immunizing
unit." If the protective dose was found to be one milligram, the se-
rum was designated as "10 normal." It is difficult to compare the
definitions of units as described by Washbourn and by Römer, but
their contributions demonstrated the feasibility of standardizing
antipneumococcic serum by the use of animals.

Tests of protective effect: the mouse protection test. In 1909, Neufeld and Haendel[989] described a method of testing antipneumococcic serum that was more or less generally adopted and used until comparatively recent times. Mice were injected intraperitoneally with 0.2 cubic centimeter of serum and three hours later different groups of the animals were given increasing amounts of culture by the same route. It appeared that the action of antipneumococcic serum follows the law of multiple proportions only within certain limits and under certain conditions and, furthermore, that there is a limit to the effectiveness of the serum. To this latter character was applied the term *Schwellenwert*. Ungermann and Kandiba (1912)[1436] found that when 0.2 cubic centimeter of serum protected mice against 0.1 cubic centimeter of culture, one-tenth of that amount of serum did not protect against 0.01 cubic centimeter of culture, thus substantiating Neufeld and Haendel's observations regarding multiple proportions. Neufeld and Haendel[992] reported additional technical details of their method in which varying amounts of both serum and culture were employed and recommended duplicate series of tests and the inclusion of a standard serum of known potency. Wadsworth and Kirkbride[1470] reported that more consistent results were obtained when one-half the amount of serum recommended by Ungermann and Kandiba was used, but Avery, Chickering, Cole, and Dochez[36] were in favor of adhering to the more commonly used quantity.

In 1918, as a result of a regulation requiring that control tests on antipneumococcic serums in this country be done at the United States Hygienic Laboratory (now the National Institute of Health), Wayson and McCoy[1493] reported the adoption of the Neufeld method as modified by Cole, with a serum supplied by Cole as the standard for purposes of comparison. Official control was confined to Type I serum. Because of the importance of this action in developing methods of standardization, a brief citation of the essentials of the technique is given. The test consisted in inoculating a series of mice with a fixed dose of serum and varying doses

of culture. Serum and culture were injected intraperitoneally, either simultaneously or one immediately following the other. A serum was considered sufficiently potent if 0.2 cubic centimeter protected mice weighing from fifteen to twenty grams against 0.1 cubic centimeter of an eighteen to twenty-four-hour broth culture of such virulence that 10^{-6} cubic centimeter killed control mice of similar weight. Survival of mice for ninety-six hours indicated protection. There were irregularities in the results obtained by this method, but it served as a valuable means of controlling the potency of serums by establishing minimum standards. Wadsworth, Kirkbride, and Gilbert (1919)[1472] described the New York State standard method of testing antipneumococcic serum which, in its essentials, corresponded to that of the federal laboratory. In 1919, the United States Public Health Service[1439] published the details of the mouse protection test as used at the Hygienic Laboratory.*

Felton (1924)[396] experimented with varying amounts of Type I culture in protection tests in an effort to overcome the irregular results obtained by the governmental method. He defined a unit of antibody as that amount which would protect mice against 0.5 cubic centimeter of a 1 to 10 dilution of an eighteen-hour broth culture of such virulence that 10^{-7} cubic centimeter killed mice in thirty-six to forty-eight hours. This dose was calculated to contain one million minimal lethal doses. In estimating the protective power of an antibody solution, Felton tested varying dilutions of the serum against a constant inoculum of culture in duplicate series of mice, as practiced by Neufeld (1922).[977] Formerly the test depended upon titrating a constant amount of serum against varying amounts of culture; here the reverse was true, that is, a constant dose of culture was used against varying amounts of serum. In 1928, Felton[402] presented the details of the protection test developed in his investigations. The method was an improvement over those previously used, but it was subject to limitations, recognized by Felton, which will be considered later.

* See Appendix.

In 1921, at the International Conference on the Standardization of Sera and Serological Tests in London, a committee of the Health Organization of the League of Nations[506] drew up a plan for the technique to be practiced in the assay of antipneumococcic serum. At the Second International Serological Conference,[596] held in Paris in 1922, it was believed that the titration test in mice, when controlled by a standard serum, would provide a method for standardization. Emphasis was placed upon the necessity of including a control serum in every test. In 1923, a subcommittee of the Health Organization of the League of Nations[597] proposed, as a basis for international investigation, that, in performing the mouse protection test, constant volumes of culture should be mixed for five minutes with decreasing volumes of serum and the mixtures injected intraperitoneally into mice. The method* was found in some instances to be satisfactory by Christensen,[847] but it failed to receive universal recognition.

In two articles dealing with the protective action of Type I antipneumococcic serum in mice, Sickles (1927)[1276-7] stated that if broth cultures, freed from organisms, were added to dilutions of serum and the precipitate removed prior to use in the test, the protective action of the serum was diminished or neutralized. It is probable that most, if not all, of this loss of activity was due to absorption of antibody by the soluble type-specific and species-specific substances although these were not emphasized as the factors responsible for the removal of protective antibody. It was stated, however, that the reaction appeared to be associated with that by which the precipitative elements of serum are absorbed when treated with the homologous culture broth.

Felton[406] utilized the principle in developing the technical details of the neutralization method for assaying the antibody content of antipneumococcic serum and concentrates. In the test, equal amounts of a diluted, neutralizing culture and varying serum dilutions are mixed and incubated at 37.5° for one to two hours. The

* See Appendix.

neutralizing antigen is made by adding 0.4 per cent formalin to an eighteen-hour serum-broth culture of virulent pneumococci of the type desired. It is necessary to allow a sufficient period of contact with formalin to elapse in order to kill the organisms. The proper dilution of the neutralizing culture is determined by a preliminary test with the control serum. Mice are injected intraperitoneally with 0.5 cubic centimeter of the serum-neutralizing culture mixture and with 0.5 cubic centimeter of a 1 to 200 dilution of a three to six-hour culture made from an eighteen-hour transplant of a mouse passage culture. Mice used to test the virulence of the organism are also injected with 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} cubic centimeter of the living culture, and blood-agar plates are made of these dilutions for counting the number of pneumococci present. The potency of a serum is determined by comparing the highest dilution of serum that will protect for twenty-four hours at least two of the three mice injected with the highest dilution of the control serum conferring a similar degree of protection.

One of the many difficulties encountered in mouse protection tests has been a lack of uniform results with samples of serum tested in different laboratories, and even when tested repeatedly in the same laboratory. At least one source of the discrepancies has been the fact that too few mice were used on each dose of serum or culture. Recognition of this fault resulted in various changes in the test, and the Park and Cooper[1053] modification of the Felton technique has served as a basis for the mouse protection test commonly used in this country at the present time.

In February, 1933, the Committee on Standardization of Antipneumococcic Serum of the Biological Section of the American Drug Manufacturers' Association suggested a method based on the Park-Cooper modification of the mouse protection test. The method was revised in December, 1933, and in its essentials has been approved by the National Institute of Health which supplies a standard bivalent serum for comparison in Type I and Type II protection tests. In general, the method conforms to that of Felton already described. For establishing the unit value of an un-

known serum, at least two complete tests must be made, using ten mice for each serum dilution. Different laboratories have modified the details of the test, particularly with regard to the doses of serum and culture employed. It has been found that satisfactory results may be obtained by using dilutions of serum and culture higher than those proposed in the American Drug Manufacturers' Association method. The details of a similar modification which, in the experience of the authors, has proved satisfactory may be found in the Appendix, pages 651–653.

Limitations of the mouse protection test. Although the mouse protection test is generally accepted as the most satisfactory means of assaying antipneumococcic serum for therapeutic use, it has been subjected to criticism on the basis of its inaccuracy and the irregular results not infrequently obtained. Aside from the personal equation in the technical manipulations of the test, the most important variables are those connected with the cultures and the mice used and the ratio between the amounts of serum and culture injected.

Variations related to the culture may be referable to fluctuations in the virulence of the organisms, their ability to neutralize or combine with antibody, and the number of pneumococci present in the test inoculum. Following a study of the standardization of antipneumococcic serum, Enlows (1923)[366] stipulated that the culture should be used in its active or early stationary phase of growth. Felton (1925)[397] observed that the unit value of serum might vary depending upon the strain of Pneumococcus employed in the test, even though only slight differences in virulence of the cultures could be demonstrated. Variable results occurred also when the same strain of Pneumococcus was used under standard conditions. According to Felton,[402] the change from larger to smaller doses of organisms gave more satisfactory results in protection tests.

In studies dealing with the lethal power of the test culture in potency tests, Morgan and Petrie (1931, 1933)[917, 1085] concluded that some control over this factor could be obtained by choosing

suitable mediums for growth and by selecting appropriate doses of culture. In considering the variables in the mouse protection test, Felton[405] placed the greatest emphasis on the culture employed and cited the killing power and antibody-neutralizing ability of the organisms as factors of first magnitude. There can be no doubt that variations in cultures contribute to lack of uniformity in the results of tests. Although many laboratories employ the so-called original Neufeld Type I strain, conditions under which cultures are maintained are certain to induce changes of greater or lesser extent in the organisms. In order to minimize variations in cultures of the various types used it would be well to have an international central supply from which representative strains could be issued and to establish standard conditions under which cultures should be maintained in different laboratories. A similar suggestion was made by Felton and is worthy of general consideration.

Variability in the outcome of potency tests on antipneumococcic serum is introduced by the mice employed. Uniformity in susceptibility, the numbers used for each determination, the age, weight, and sex of the animals are all factors to be considered. Petrie and Morgan found that the sex of the animals appears to have no significant influence on the results of titrations. Furthermore, the weight of mice had no effect on the mortality-rate, but heavier animals tended to show a longer survival time. Although little work relating to the effect of age of mice has been done, it is preferable to use young stock. Of greater importance is the degree of uniformity in susceptibility of the mice to pneumococcal infection. It has been customary in titrating serum to employ, when possible, white mice from homogeneous breeding stock. Even when this is done, some mice, or groups of mice, may exhibit unequal susceptibility to the infecting doses of culture. Petrie and Morgan found that from 5 to 10 per cent of the animals used possessed an innate resistance to small doses of pneumococci.

In attempts to decrease the discrepancies introduced by the test animal, it has become customary to increase the number of mice used per dose of serum. In the older methods where only two or

three animals were used for each amount of serum or culture, chance death or survival caused confusion. Trevan (1930)[1418] found that the number of animals necessary to offset a given margin of error depends on the ratio of the increment of effect of the serum to the increment of dose of culture. As an example, it was found that if forty mice per dose are used to test two serums, the first may be estimated as equal in potency to the other, although in reality the potency of the first may be as low as 72 per cent or as high as 140 per cent of the second. Trevan suggested computing the survival time of test mice as a means of increasing the accuracy of results and believed that by this plan not more than one-half the number of mice would be required for the same determination as would be necessary if simple enumeration of the number dead on a given day were made. It was found, however, that this plan might be unduly favorable to the serum, since the distribution of survival times was not normal. Discrepancies in the results of tests were also investigated by Beard and Clapp (1932),[95] who recommended the use of at least ten mice per dose of serum and at least two independent tests on each lot of serum. Results of different tests were considered together, rather than separately. Barnes, Clarke, and Wight (1936),[82] using the method described in the Appendix, found that when two or more separate tests, each of which included thirty mice per sample of serum, were used in estimating the value of a serum as compared with a standard, it was practicable to arrange the amounts of serum in the ratio 4 to 2 to 1.

Calculations of the potency of serum were based on the following equation:

$$\text{Units per cubic centimeter in unknown serum} = a \left(\frac{Dx}{D'y} \right)$$

a = units per cubic centimeter in control serum
D = total number of mice dead on control serum
x = lowest dilution of unknown serum
D' = total number of mice dead on unknown serum
y = lowest dilution of control serum

Although this method served to compensate for irregularities in the mortality-rate of the test animals, it has been found to be unreliable, since a different value may be obtained when the number of survivals is substituted for D and D'. A more accurate formula has been derived and is included in the Appendix, page 657.

It has been claimed that satisfactory end points may be obtained by using serum doses more closely spaced (Park, 1930;[1058] Morgan and Petrie, 1933[917]), but in the experience of the authors of the present volume this plan is not reliable under routine laboratory conditions unless an inconveniently large number of mice are used.

In a recent series of studies on the mouse protection test, Goodner and Horsfall[537] and Goodner and Miller (1935)[540] decided that the most important variable in the mouse is the number of monocytes present in the peritoneum at the time of injection of serum and culture. The mice were carefully selected from a strain inbred for several years. It seems not unlikely that the phagocytic activity of the animals used may condition the progress of the pneumococcal infection in the protection test. On the other hand, it is questionable how this factor may be controlled in selecting mice, even from an inbred stock, at the time of carrying out protection tests. Irwin and Hughes (1931)[670] found in studying the bactericidal power of the blood of rats that, when matings were made of susceptible animals, that is, those with low bactericidal powers, the offspring were all susceptible; if a low $\times$ high mating was made, two-thirds of the offspring were susceptible and the remainder resistant. The breeding of a stock of mice of uniform susceptibility to pneumococcal infection, therefore, would appear by analogy to be possible. Such a program has been initiated at the Rockefeller Institute under the direction of Webster and by Little at Bar Harbor. If the efforts are successful, it may be possible to establish a supply of mice of uniform susceptibility for titrating antipneumococcic serum.

The outcome of protection tests is also dependent upon the pro-

portions of serum and culture employed. The so-called *Schwellen-wert*, also noted by Neufeld and Haendel,[989] is another phenomenon which limits the applicability of the test. The *Schwellenwert* may be defined as the maximal amount of culture against which protection can be demonstrated with any amount of serum. Coventry (1927),[286] in measuring the protective action of serum by noting survival time of the mice, failed to obtain evidence of this threshold of effectiveness of serum. That such a relation exists, however, appears to be generally conceded although the explanation is obscure. Goodner and his colleagues[537, 540] believed the phenomenon to be due to the interrelation between serum and culture and the number and kind of phagocytic cells in the host. The authors also list certain zonal effects as referable to the variable factors in the test. Thus, the limiting zone of titration is defined as the smallest amount of serum that will protect against a given quantity of culture, and is believed to be related to the number of virulent organisms and to the antibody content of the serum. The reaction, however, is not strictly one following the law of multiple proportions. In the same general category is the so-called pro-zone which is manifested by a lack of protection when an excessive amount of serum is employed. The pro-zone is, therefore, related to the *Schwellenwert*. Goodner and Horsfall[537] found such a zone in tests with immune horse serum but not with immune rabbit serum, and others have confirmed this observation.

The fact that the zonal effect may be observed in tests with horse serum but not with rabbit serum suggests the presence of some non-protective, antagonistic substance in immune horse serum. The effect may be at least partially obviated according to Goodner and Horsfall by previous treatment of the mice with sodium nucleinate. That excessive amounts of specific polysaccharide may serve to operate against the protective action of serum is a possibility suggested by the work of Felton and Bailey (1926).[419] The interference may be due simply to a combination of polysaccharide and antibody, thus decreasing the amount of anti-

body available for protection. Felton[402] suggested that an action of this nature might be responsible for the zonal effects mentioned above, or it is possible that Goodner and Horsfall's explanations already cited may be nearer the truth.

Confusion in the standardization of antipneumococcic serum has been occasioned by the lack of a universally accepted control serum. The need was supplied by Cole in 1918, and Felton (1930)[405] distributed serum "F 146" to many laboratories as a standard for tests on Type I and II serum. The supply was not large, however, and efforts were made in this country and abroad to substitute other serums comparable in potency to F 146. Unfortunately, the present standard serum for the United States (as well as F 146) is supplied in liquid form and, according to Hartley and Smith (1935),[596] is subject to deterioration. It has been the experience of the authors that loss of protective power of a control serum may introduce major difficulties in standardization. Both F 146 and P 11, the present American standard serum, are for Type I and II titrations. Hartley and Smith have proposed an international standard serum, to be distributed in dry form, for Type I and another for Type II. The unit values of these standards are based on Felton's F 146. Hartley and Smith have placed their standard serums at the disposal of the Health Organization of the League of Nations. If present methods of standardization are continued in use, it follows that similar standards for types other than I and II will be required.

Tests of therapeutic effect. One of the objections to the protection test as generally employed in mice has been the fact that it is a prophylactic test rather than a means of estimating the therapeutic activity of serums. Experience has indicated that the curative value of serums in human beings may be expressed in terms of mouse protective units, yet there has been a surmise that products selected on the basis of experimentally demonstrated therapeutic efficacy might yield better results in treatment. Attempts have therefore been made to devise tests in both rabbits and mice for de-

termining the curative properties of serum in pneumococcal infections.

Washbourn[1487] administered a single dose of serum to rabbits five to six hours following inoculation of a test dose of culture and observed protection, but when the serum was given eight to twelve hours after the culture he observed only retardation of death. Wadsworth[1458] utilized rabbits in testing the curative action of immune rabbit serum, but used the method for experimental comparison rather than for actual standardization. In a series of publications beginning in 1928, Goodner[525] reported experiments dealing with the therapeutic effect of antipneumococcic serum in rabbits following intradermal infection. By inoculating rabbits intradermally with small amounts of living, virulent pneumococci it was found that a local lesion developed and symptoms appeared somewhat analogous to lobar pneumonia. The results of intravenous treatment of animals so infected with antipneumococcic serum administered twenty-four hours after inoculation were advanced as a means of estimating therapeutic efficacy. The curative value of serum as determined by the Goodner technique did not correspond with the results of the mouse-protective test. The intradermal method was suggested (1929)[527] for the routine standardization of serum since it was based on the ability of a serum to combat a preexistent infection. Later (1933),[531] however, it was admitted that the method was too elaborate for routine use, and it was found (1934),[532-4] furthermore, that variable factors, such as the severity of the infection, the number of white cells, and the breed and weight of the test animals complicated the results. In the observations of Francis and Terrell (1934)[476] on experimental Type III intratracheal infection in monkeys, it was found that the mortality was dependent upon the presence and severity of the septicemia following inoculation. Thus, there were no deaths in animals without bacteriemia; with a low degree of blood invasion the mortality-rate was 45 per cent; with a moderate degree of septicemia 75 per cent died; and with massive septicemia all animals died.

In intradermal infections in rabbits with Type I pneumococci, it appears that similar mortality-rates may be expected. Barnes and Clarke, in investigations* (1934) on the intradermal method, observed extremely wide variations in the number of pneumococci present in blood samples taken twenty-four hours after infection. Because of the time at which serum treatment of rabbits is ordinarily begun in this method, it is impossible to base dosage on the severity of septicemia, inasmuch as this condition cannot be readily determined until twenty-four hours after taking blood samples. As a result of this and other objections (irregularity in susceptibility to infection, et cetera),[531, 533] the intradermal method of assaying antipneumococcic serum is impracticable for routine use.

Tests for the curative effects of antipneumococcic serum in mice have also been advocated. Coventry[286] reported that a majority of mice could be saved by administering concentrated serum intraperitoneally four hours after an injection of Type I culture had been given by the same route. Parish (1930)[1051] studied the therapeutic effect in mice of serum given intravenously at intervals of one and one-half to seven hours after the intraperitoneal injection of culture. It was found that serum was effective when given in this manner but, as was to be expected, the longer the interval between injections, the fewer the animals that survived. Petrie and Morgan[1086] reported investigations on the curative action of antipneumococcic serum in mice and stated that in the case of Type I recovery might take place even when a bacteriemia was present. The serum was administered intravenously up to twelve hours after inoculation. The outcome of the test was apparently conditioned by the amount of antibody injected, the time of its injection in relation to the infecting dose, and the degree of infection at the time of serum administration. The authors thought that the test might prove satisfactory as a means for standardizing therapeutic serum.

In a later communication, Morgan and Petrie[917] observed that a comparison of the curative and protective tests showed that both

* Unpublished.

were equally reliable for measuring the protective antibody, but that the prophylactic technique was more convenient. Trevan[1418] found that intravenous administration of serum did not increase the accuracy of titrations. It appears, therefore, that curative tests on mice for estimating therapeutic value of serum fail to offer any information in addition to that obtained by the usual protection tests and, because of their greater inconvenience, cannot be considered as of sufficient worth to warrant general adoption.

Tests of other effects. During the development of antipneumococcic serum therapy, investigators have sought for qualities in serum other than those of an antibacterial nature. Attempts have been made to demonstrate antitoxic properties (Klemperer brothers[724]), but no convincing evidence has yet been brought forward that pneumococcal antitoxin has been produced. The work of Clowes, Jamieson and Olson (1926),[244] of Parker and McCoy,[1062] and of Coca[246] has already been discussed in some detail in other chapters, but as yet none of the methods described has been adopted for the routine testing of therapeutic antipneumococcic serum.

With the Goodner intradermal technique, Curphey and Baruch (1930)[292] tested the therapeutic effect in rabbits of so-called exudate antiserum. When compared by mouse protection tests, the preparations showed essentially the same antibody content as serum obtained from horses immunized by formalinized vaccines. When compared in rabbits for therapeutic effect, however, it was found that 70.4 per cent of animals treated with the "exudate antiserum" survived, while only 34.7 per cent of those given the usual antipneumococcic serum lived. As a result, the authors assumed that "exudate antiserums" contain substances, other than antibacterial antibodies, which exert greater therapeutic action.

Sabin[1204-6] reported experiments which led him to conclude that the therapeutic action of antipneumococcic serum depends to a considerable extent on a non-antibacterial factor. He found type-specific protective antibody in serum after complete precipitation

of the anticarbohydrate precipitins, using capsular polysaccharide prepared by the early method of Heidelberger, Goebel, and Avery.[613] The soundness of the argument appears questionable in view of the more recent experiments of Avery and Goebel (1933)[46] with acetyl polysaccharide. Ward (1932),[1483] after using the sensitive bactericidal test in determining the phagocytic power of SSS-absorbed serum, concluded that there was no necessity to predicate the presence of an additional and possibly non-antibacterial antibody in immune serum. Other evidence advanced by Sabin to support his hypothesis depended upon tests with serum prepared or utilized in different ways in treating the dermal pneumococcal infection in rabbits, but the results failed to parallel those of the mouse protection tests.

Some antipneumococcic serums are therapeutically more active than others and the differences may possibly be due to the presence or absence of antitoxic or non-antibacterial substances in addition to the mouse protective antibody. Felton,[404] however, doubted the evidence put forward in support of this hypothesis. Claims for extra-therapeutic properties of serum based on tests in rabbits infected intradermally are subject to the limitations of the method.

IN VITRO TESTS

Various means have been employed for titrating the antibody content of antipneumococcic serum without resorting to the use of animals. Thus, tests for determining the amount of water-insoluble proteins, the complement fixation and bactericidal reactions, flocculation with toxic preparations, and the *Quellung* reaction, in addition to agglutination and precipitation tests have been devised for the purpose.

Water test. The so-called water test was an outgrowth of the observation by Felton (1924)[396] that simple dilution of serum with water resulted in precipitation. It was thought that the amount of precipitate so formed was an indication of the protective strength of the serum and, furthermore, that the method could be used to

determine the suitability of the serum for concentration (Goodner[528]). The method, however, has not proved to be accurate and has fallen into disuse.

Complement fixation. Although there appear to have been no systematic studies directed toward applying complement fixation as a means of serum standardization, it is a method which, at least from the theoretical standpoint, might be employed.

Bactericidal power. Tests for determining the bactericidal or phagocytic power of blood have been developed in attempts to measure natural or artificial immunity to pneumococci. However, because of irregularities in the results, tests for bactericidal action have not been adopted routinely for the standardization of therapeutic antipneumococcic serums.

It is generally assumed that defense of the body against pneumococcal infection depends on the neutralization of capsular polysaccharide, thus permitting phagocytosis of the organisms. Determination of the opsonic or tropic activity of serum should presumably offer a valuable means of estimating therapeutic effect. No *in vitro* method of this sort yet devised is superior to a satisfactory mouse protection test.

Antitoxic flocculation. If it is proved that antitoxic properties can be imparted to antipneumococcic serum and if these properties are of clinical benefit, then some *in vitro* method for estimating the antitoxic potency of the serum will be needed. In that event, the flocculation test devised by Powell* would be applicable.

Quellung reaction. Clapp, Phillips, and Stahl (1935)[234] have suggested an adaptation of the well-known Neufeld *Quellung* reaction for the titration of antipneumococcic serum. The technique requires relatively small amounts of materials and appears to be equally applicable to all types of antipneumococcic serum. Some of the difficulties involved in other methods are apparently avoided in this test, but its advantages as a routine procedure have not as yet been demonstrated.

* Mentioned by Clowes, Jamieson, and Olson.[244]

Agglutination. Agglutination tests have been utilized in the past for measuring the antibody content of antipneumococcic serum. In view of the more recent work on the subject (Heidelberger and Kabat, 1934[614]), it appears that agglutination tests may give a definite measure of the antibody response of animals but owing to uncontrollable variables the usual method does not yield sufficiently consistent results to justify its use as a routine means of standardizing serums.

Precipitation. Accumulated evidence strongly indicates that the effective therapeutic antibody in antipneumococcic serum is closely related to, if not identical with, the immune substance reacting with the type-specific capsular polysaccharide. The logical course in establishing *in vitro* methods of standardization is, therefore, to utilize tests employing the specific carbohydrate of the various pneumococcal types as precipitinogen. Although precipitin reactions between antipneumococcic serums and corresponding antigens were observed soon after the development of immune serum (Panichi, 1907[1046]), it was not until Heidelberger and Avery[606] isolated the capsular polysaccharide that efforts were made to apply specific precipitation methods in the standardization of antipneumococcic serum. With many of the tests carried out with polysaccharides similar to the original preparations of Heidelberger and Avery, the results have been decidedly promising. It is probable, however, that even more accurate and reproducible titrations may be obtained with the acetyl form of polysaccharide more recently described by Avery and Goebel (1933),[46] or better still, with the purified materials prepared according to the later methods of Heidelberger, Kendall and Scherp.[627]

In general, there have been three methods employed for determining the precipitin content of antipneumococcic serum: one using a constant amount of polysaccharide mixed with varying dilutions of serum; another utilizing the principle of optimal proportions of antigen and antibody; and, finally, one dependent

upon the determination of the nitrogen content of antigen-antibody aggregates.

The first-named procedure, which may be termed a routine precipitin test, has been subjected to various modifications and interpretations. With this method, Zozaya, Boyer and Clark (1930),[1589] Felton (1931),[407] Falk, McGuire, Valentine and Whitney (1931),[387] and Barnes, Clarke and Wight[82] obtained fairly consistent results in titrating whole antipneumococcic serum. Inaccuracies are introduced by variations in spacing of dilutions of serums and in establishing end points, in addition to variations in technical manipulations. It is customary to take as the end point the highest dilution of serum affording visible precipitation with a fixed amount of antigen after incubation for two hours in a waterbath at 37.5° and storage overnight in the cold, although Zozaya, Boyer, and Clark preferred to make readings after the preliminary two-hour incubation. If the spacing of serum dilutions is too wide, the titrations will not be satisfactory for comparative purposes. On the other hand, difficulty in reading end points arises when the increment of antibody is too small. Barnes, Clarke, and Wight used the same serum dilutions employed by Sobotka and Friedlander (1928)[1299] which proved satisfactory in routine tests. The details of the method used by Barnes and his associates are given in the Appendix, page 644.

The importance of optimal proportions of serum and antigen in precipitin reactions was pointed out by Dean (1911),[310] and reported upon in detail by Dean and Webb (1926).[311] Morgan (1923)[912] studied the principle in relation to antipneumococcic serum, and further observations were reported by Smith (1932),[1298] who stated that titration by the method would fulfill the requirements placed upon an *in vitro* method of testing native serum. Unfortunately, the results with concentrated preparations were not wholly satisfactory. The method is similar in principle to the flocculation test introduced by Ramon (1923)[1120] for assaying diph-

theria antitoxin, and since the technique is given in the Appendix, its details need not be described here. Smith used a small number of samples in developing this method as applied to antipneumococcic serum, but Barnes, Clarke, and Wight[82] corroborated his findings in titrations on a larger series of unconcentrated Type I and II serums.

In 1935, Felton and Stahl[432] described a method of titration by determining the combining equivalents of antibody and SSS. The method is based on the principle of optimal proportions, and the point of equivalence is measured by testing for excess antigen in a series of mixtures of antigen and antibody, rather than by determining the point at which particulation first occurs as in Smith's method. The method reported by Felton and Stahl appears to be applicable to concentrated antibody preparations as well as to native serum and this fact may favor its adoption in the event that *in vitro* methods of standardization are eventually substituted for mouse protection tests.

A different method of estimating the antibody content of serums was introduced by Heidelberger, Sia, and Kendall (1930),[630] who calculated the amount of immune nitrogen in SSS-antibody precipitates and converted this figure into terms of immune protein present in the serum. Additional details of the method and results of titrations have been reported in subsequent publications.[619, 624] The essentials of the technique may be found in the Appendix, pages 643 and 644.

CORRELATION BETWEEN IN VITRO AND IN VIVO TESTS

Much of the evidence obtained thus far concerning the relationship between laboratory tests and therapeutic efficacy of antipneumococcic serum has been based on mouse protection tests, and the correspondence has proved to be satisfactory for general purposes. If the mouse test is to be supplanted by a chemical or serological method of standardization, the correlation between the results obtained by mouse protection tests and various *in vitro* methods of assay must be determined. The best approach to this

question is a study of the relation between mouse protection and the amount of polysaccharide antibody as determined by some form of precipitation test. Brown (1933)[154] found a high degree of correspondence between mouse protection and precipitation with the cellular carbohydrate, but it is difficult to concede any advantage in its use over the purified forms of capsular polysaccharide.

In a study of thirty-nine serums, Felton[407] observed correlation coefficients of 0.93 and 0.91 between protection and precipitin titer, and between protection and the amount of immune protein precipitated with polysaccharide. Barnes, Clarke, and Wight, using fifty samples of unconcentrated serum, calculated the correlation coefficients between mouse protection and their modified routine precipitin test, Felton and Stahl's combining equivalent test, and Smith's optimal proportions test. Correlation coefficients found for these three methods were 0.930 ± 0.012, 0.925 ± 0.014, and 0.909 ± 0.016, respectively, in the case of Type I preparations. Between mouse protection and the first two precipitin tests, values of 0.817 ± 0.031 and 0.739 ± 0.043 were obtained in the case of Type II serums. Felton and Stahl[432] reported correlation coefficients of 0.90 and 0.89 for Type I and Type II, respectively, between the combining equivalent test and mouse protection for both native serum and concentrates. Results of titrations by methods depending upon precipitation of antibody by SSS therefore agree closely with those of the mouse protection test. It is not unlikely that the correlation would be even higher were it not for the inherent errors of the mouse protection test.

The data on methods of standardization may be summarized thus: The mouse protection test is inconvenient, expensive, and subject to variations difficult to control. It appears likely that some *in vitro* method of titration may ultimately supplant the animal test, and of those discussed, some form of precipitin test with purified capsular polysaccharides offers the greatest promise. The adoption of any new method depends largely upon its simplicity, accuracy, and close correlation between estimated potency of the

preparations tested and their therapeutic efficacy. Moreover, the method should be equally applicable to unconcentrated and concentrated products. From the theoretical standpoint, at least, some method utilizing the principle of optimal proportions appears to meet these requirements. As long as the mouse protection test remains the accepted procedure for assaying antipneumococcic serum, a stable, standard serum should be available for general use, only standard cultures maintained under uniform conditions should be employed in the test, and a technique should be adopted that would be acceptable to all engaged in the manufacture and use of the products.

Confusion has existed because of a lack of any satisfactory definition of a unit of antibody. The unit as defined by Felton (1924)[396] furnished a convenient working basis, but experience has shown that the number of lethal doses of pneumococci against which a given serum will protect is too variable a factor to serve as a criterion for establishing the unit. A stable, desiccated standard serum of which a given weight contains, by international agreement, one unit of antibody as suggested by Hartley and Smith,[596] would, for the time being, supply the need. In the event that a precipitin test is substituted for the mouse protection method of standardization, it is possible that the standard of comparison, as suggested by Felton and Stahl, should be a uniform and satisfactory preparation of type-specific polysaccharide, rather than a control serum.

A comprehensive discussion of the standardization of antipneumococcic serum and concentrates is to be found in the latest communication of Felton and Stahl (1937).[433] The report comprises the results of a restudy of the various methods advocated for determining the potency of serum. The original report should be consulted for the details of the several tests carried out in the study but, because of the pertinency of the conclusions, some of them are quoted here.

(1) When the mouse-protection test is applied by the technic described in this paper, namely by carrying out the test in the zone in which the law of multiple proportions is valid; by controlling the dose of culture by preliminary test against 0.5 unit of antibody; by using mouse strains which have been shown to be uniform in their resistance to pneumococci and by titrating the unknown serum or concentrate against the protective power of 0.5 unit of antibody of a standard control serum, reliable and reproducible assays of the protective titer of types I and II antipneumococcus serum can be made.

(2) The in vivo method described in this report termed the "combining-equivalent method" which, in principle, measures the antibody present by means of the amount of SSS with which it combines shows a high degree of correlation with the mouse-protection test described in the foregoing (in 62 samples $r = 0.94$ for type I and $r = 0.91$ for type II antibody).

(3) The use of the "combining equivalent technic" as an economical, rapid, and reliable method for the titration of antipneumococcus serum is suggested.

(4) Standardization by several different methods in 1933 of the United States National Institute of Health's standard serum P11 by comparison with serum F146 gave the following results: Mouse-protection method, 300 type I units and 131 type II units per cc; by the neutralization method, 333 type I and 150 type II units per cc; by the combining equivalent method, 322 type I and 150 type II units per cc; and by Heidelberger's method, 308 type I and 150 type II units per cc.

(5) Standardization by three different methods in 1935 of three sera—F146, Lyo P11, and the British type I and type II dried standards, with proof of the deterioration of serum F146 as compared to serum P11, gave the following results for the antibody content of the British standards as compared to serum P11: Mouse-protection method, 1,150 type I units, 750 type II units; by the combining equivalent method, 1,177 type I units, 720 type II units; by Heidelberger's method, 949 type I and 426 type II units per cc of the respective samples.

Safety Tests

Antipneumococcic serum intended for therapeutic use must be tested for the possible presence of harmful substances. The most important tests are those planned to demonstrate freedom from

tetanus spores and from pathogenic bacteria, the absence of toxic or chill-producing properties, and an excess of preservative. The National Institute of Health[1440] requires that "a sample of each lot of all products shall be tested for identity if such test is applicable, otherwise for safety, after the labels have been affixed to the final containers."

ROUTINE SAFETY TEST

This test consists in the injection of 5.0 cubic centimeters of the product into the peritoneum of a normal guinea pig. The animal is observed over a period of ten days for any symptoms referable to harmful serum components or to bacterial contamination.

The possibility that the intravenous administration of antipneumococcic serum may result in more or less severe thermal reactions renders it obligatory to test each lot for this property. The tests proposed by Sabin and Wallace[1209-10] in dogs, and by Barnes and Kramer,[83] and Barnes and Robinson[84] in monkeys have been discussed in a previous section of this chapter. It would be desirable to test for this property on human subjects, but where such a course is not possible, one of the two animal tests mentioned should be employed. In addition to chill-production, other unwanted properties of the serum may thus also be detected.

TESTS ON MICE

One of the reasons advanced by Felton and Stahl for continuing the use of the mouse protection test in standardizing antipneumococcic serum is that it also serves as a safety test. The value of the test, however, is limited; mice and rabbits are unreliable animals for detecting chill-producing properties, and the usual mouse protection test is of insufficient duration to meet the requirements in other respects to ensure the safety of the serum.

Mice may be used for detecting excessive amounts of preservatives as described by Leake and Corbitt.[794] The need for such a test rarely arises, but the intraperitoneal and intravenous injection in

mice affords a rapid and convenient means of detecting the presence of toxic amounts of some of the chemical preservatives used in serum.

Final Processing of Serums

After a lot of antipneumococcic serum has been selected and, as is the case in many laboratories, has been concentrated, there are routine procedures to be followed before the product is ready for distribution. A brief discussion of a convenient and approved system covering this phase of processing follows.

TOTAL SOLIDS

It is necessary to determine the total amount of solids present in concentrated preparations intended for human use, because the solids content may be too high to permit of ready filtration and ease of administration. The determination is made by drying to constant weight an accurately measured amount—at least ten cubic centimeters—of serum and calculating the percentage of dried matter in terms of the original weight of serum. Experience has shown that concentrated serum with a total solids content between 10 and 15 per cent is usually of satisfactory viscosity to meet the requirements in processing and administration to patients. Lots with a solids content above this level are diluted with an appropriate volume of salt solution.

FILTRATION

All antipneumococcic serum intended for therapeutic use must be passed through a Berkefeld or some similar filter to ensure the absence of contaminating microorganisms. Filtration of concentrated products is facilitated by first passing the serum through a high-speed centrifuge. Warming the serum to slightly above room temperature also facilitates filtration. In order to minimize subsequent testing, it is desirable to filter as large quantities of serum as possible into single containers. The usual aseptic precautions must be observed throughout the process.

BULK STERILITY AND POTENCY TESTS

As soon as the lot of serum is filtered, samples are removed for sterility and potency tests and for tests of chill-producing properties, in case the latter test is to be performed on laboratory animals. Regulations of the National Institute of Health covering the tests for sterility are included in the Appendix, page 662.

It is necessary to determine the potency of antipneumococcic serum prior to filling the final containers. These tests, as well as tests on animals for chill-producing qualities, may be done conveniently with samples taken from the bulk containers and while the tests for sterility are being made. The methods employed have been described in a previous section of this chapter. It is obvious that the product must pass these tests satisfactorily before it is dispensed into the final containers.

DISPENSING AND LABELING

After the serum has passed all the tests made on samples taken from the bulk containers, it may be dispensed into final containers for distribution. Sterile, rubber-capped vials or syringe ensembles of a capacity never greater than the maximal dose for man are used for the purpose. A convenient method of dispensing the bulk product into the vials or syringes is briefly as follows: The serum from the bulk container is forced by compressed air into a burette, equipped with a specially designed filling device, which, by means of a protecting bell or dome, covers the neck of the vial to be filled. All openings are protected against air-borne bacteria by sterile cotton air-filters. The entire filling apparatus must, of course, be sterilized before use. As soon as the vial is filled with serum, a soft rubber stopper is inserted, and sealed with a viscose cap or gelatin. The bottling process should be conducted under strict aseptic conditions in a room free from draughts and dust.

When the lot of serum has been dispensed into the final containers, approved labels, on which are recorded the lot number of the preparation, the potency for each type of antibody contained,

and the expiration date set by federal regulation are affixed to the bottles.

STERILITY TESTS ON FINAL CONTAINERS

The method required by the National Institute of Health is described in the Appendix, page 662.

SAFETY AND IDENTITY TESTS

Using the serum from one of the labeled, final containers, the routine safety test in a guinea pig is made (see page 588 of this chapter).

IDENTITY TEST

For this purpose, the simple precipitin test with type-specific pneumococcal capsular polysaccharide homologous for the types of antibody contained in the product is satisfactory.

RECORDS

It is important that complete records be made of every lot of antipneumococcic serum produced. These are conveniently kept on a separate form for each lot, suitable to the needs of the laboratory, and giving in detail all essential data on each step in the process of manufacture, with the initials of the person making each entry.

In the United States, for laboratories operating under federal license, it is necessary to submit to the National Institute of Health protocols of potency tests together with two of the final containers of each lot of serum to be distributed for therapeutic use (see Appendix, page 661).

REGULATIONS GOVERNING ANTIPNEUMOCOCCIC SERUM

In the United States, control over biological products concerned in interstate commerce is maintained by the Treasury Department through the Public Health Service which, from time to time, formulates or revises and issues regulations. Antipneumococ-

cic serum (Types I and II) is included among those products for which "the official standards of potency . . . shall be those distributed by the National Institute of Health of the United States Public Health Service."

Minimum standards for Type I serum to be submitted for official tests were established in 1919, but they need not be discussed here since they were based on methods no longer in use. In January, 1934, a regulation[1437] was promulgated to the effect that samples of all antipneumococcic serum intended for sale in interstate commerce must be submitted to and released by the United States Public Health Service before distribution. A memorandum of December, 1935,[1438] stated that after April 1, 1936, the National Institute of Health would pass for distribution only serums (Type I) showing at least 1,000 units per cubic centimeter if concentrated and at least 500 units per cubic centimeter if unconcentrated. A later memorandum effective March 1, 1937, established the same minimal potency for Type II serum.

The British Therapeutic Substances Act of 1925,[549] followed by the Statutory Rules and Orders of 1927, is similar to the United States regulations governing biological products. The Statutory Rules and Orders of 1935[551] regulate the preparation of both Type I and Type II serums.

Governmental control over therapeutic antipneumococcic serum is desirable for uniformity in standardization and in interpreting clinical results. Ideally, however, it would be preferable to secure international agreement for a set of standard regulations without interference with separate governmental requirements.

AUTHOR'S NOTE: The 1936 (11th) edition of the United States Pharmacopoeia[1087] gives official recognition only to Type I antipneumococcic serum.

The Production of Diagnostic Serum

In order that patients with lobar pneumonia may be given effective serum treatment, it is necessary first to determine the infecting type of Pneumococcus. Before the serological differentia-

tion of pneumococci into groups was accomplished by Dochez and Gillespie, serum therapy of lobar pneumonia was purely empirical owing to the lack of exact knowledge concerning the diversity of pneumococcal strains. The separation of pneumococci into thirty-two distinct types and the recent development of the Neufeld *Quellung* reaction as a method of type determination have rendered serum therapy far more rational, effective, and economical than formerly. For many years, monovalent, immune horse serum was employed in the serological diagnosis of pneumococcal types, but now the Neufeld technique employing immune rabbit serum for the demonstration of capsular swelling yields more satisfactory results, besides being more rapid than the agglutination or other reactions involving the use of immune horse serum.

IMMUNE HORSE SERUM

Diagnostic serums may be produced by immunizing horses with type-specific strains of pneumococci by the same methods followed in the production of therapeutic serum. In laboratories where monovalent therapeutic serums are manufactured it is convenient to select bleedings, usually the earlier ones, and, if tests are satisfactory, to utilize the serum for type identification. In determining the suitability of immune horse serum for diagnostic purposes, the specific agglutinin titer, the presence of heterologous agglutinins for other pneumococcal types (particularly those of closely related types), and the velocity of the agglutination reaction are to be considered. For these tests it is essential to use organisms of maximal virulence, and preferably young, living cultures. The macroscopic method of agglutination is satisfactory, and readings should be taken at frequent intervals during the first two hours. Serums that are suitable for diagnostic use usually give clear-cut, specific agglutination in relatively high dilutions after one-half-hour's incubation in a water-bath at 37°, and exhibit no visible agglutination with representative strains of other types, although serums that show some non-specific agglutination in dilutions well

below the type-specific titer may be employed. If the serums are to be used for precipitation tests, the same routine should be followed in determining the titer against homologous and heterologous polysaccharide.

It is generally conceded that antipneumococcic horse serum is unsatisfactory for use in the Neufeld *Quellung* reaction because of the non-specific effects caused by that serum (Neufeld and Etinger-Tulczynska, 1931;[984] Etinger-Tulczynska, 1933;[868-a] Sabin, 1933[1206]). The British Therapeutic Trials Committee of the Medical Research Council (1934)[887] concluded, however, that results with either immune horse or immune rabbit serum are equally reliable provided the serum has an agglutinative titer of 1 to 160 to 1 to 320. At the Massachusetts Antitoxin and Vaccine Laboratory it has been observed that, even when the same vaccines are used for immunization purposes, immune horse serum is likely to give cross-reactions, whereas immune rabbit serum is highly type-specific.

Relatively small amounts of serum are required for the Neufeld test, and, therefore, it is better to use rabbit serum because of the irregular reactions exhibited by horse serum. For routine identification tests, and for other methods involving macroscopic agglutination or precipitation, diagnostic horse serum may be found more economical to use than rabbit serum.

IMMUNE RABBIT SERUM

Various methods are employed for producing antipneumococcic rabbit serum for use in the Neufeld *Quellung* test. For the purpose, methods outlined by Barnes and White[86] have given satisfactory results. Cooper and Walter (1935)[275] published the details of the methods employed at the laboratories of the New York City Department of Health and emphasized the necessity of using scrupulously clean syringes for antigen injections, and of selecting representative strains of pneumococci for immunization. It was

also found that long-continued immunization of the stock animals resulted in the acquisition by the serum of non-specific immunological properties. The latter point has been considered in the development of the so-called "rapid method" of producing rabbit typing serums, in which the animals are subjected to an intensive course of immunization and, when the titer of the serum is satisfactory, the animals are exsanguinated. Experience at the Massachusetts laboratory has shown that, with properly selected cultures killed by heating at 100°, rabbits may be immunized over a period of at least a year and bled routinely every three weeks after a course of twelve to fifteen injections. The amount of serum obtained when the plan is followed reduces the cost of production and, when the specificity of closely related strains is carefully controlled, as suggested in the paper by Barnes and Wight,[88] cross-reactions with the serum are avoided. The methods recommended by the authors are described in the Appendix, page 663.

Rabbit serum intended for use in the Neufeld method should be tested for both potency and type-specificity. Cooper and Walter (1935)[275] recommended a minimal agglutinative titer of at least 1 to 200, with freedom from cross-reactions, and that each serum be tested for its ability to cause a characteristic capsule swelling of pneumococci of the homologous type.

Some workers have maintained that rabbit serum for use in the Neufeld test must be used in undiluted state. The claim is not necessarily valid, however, for in the authors' experience completely satisfactory results are obtainable, at least with certain pneumococcal types, if highly potent serums are diluted as much as 1 to 5 to 1 to 10 with normal rabbit serum.

As in the case of therapeutic serums, it is recommended that standard methods of producing diagnostic serum for the type determination of all known pneumococcal types should be universally adopted and put into effect. A movement to bring about the fulfilment of the object has been initiated in the United States.

PRESERVATIVES, BOTTLING, AND LABELING

The choice of a preservative for diagnostic serums is not so important as in the case of therapeutic products. A satisfactory procedure is the addition of tricresol to a concentration of 0.3 per cent at the time of the collection of the serum from the clot. Any resulting precipitate or detritus may be removed by centrifugation or filtration prior to filling the serum into final containers.

Containers of diagnostic serum should be labeled with the lot number, the serological type, the dilution to be used (if any), a notation of any cross-reactions produced, and an expiration date beyond which no reliance can be placed upon the indicated potency. Serums should be retested at intervals of three to four months.

Summary

The main points developed in this chapter may be summarized as follows. For stimulation of antipneumococcal immune substances in animals, vaccines made from highly virulent, type-specific cultures, killed preferably by heating at 100°, have given satisfactory results. The so-called "three-week" schedule of injections is to be recommended, and the dosage of antigen should be as small as is consistent with satisfactory immunological response as indicated by repeated potency tests on the serum. Concentrated and refined serum is preferable to unconcentrated serum for therapeutic use; the method of Felton in which alcohol is used as a precipitant being at present the one most generally employed. Current methods for determining the potency of serums are not wholly satisfactory, but as long as the mouse protection test remains the accepted procedure, for the sake of uniformity this method should be used. A standard serum for comparative purposes should be adopted for use in the assay of antipneumococcic serum and, in the event that a precipitin test is substituted for the *in vivo* method, a standard preparation of type-specific pneumococcal polysaccharide should be available. Standard methods for preparing and test-

ing diagnostic serums for all pneumococcal types with a central source of supply and control of the serums should also be incorporated in a plan for developing the uniformity of all antipneumococcic serum.

SERUM TREATMENT OF LOBAR PNEUMONIA

The theoretical basis for the administration of specific immune serum in the therapy of pneumonia; a discussion of complicating problems and of the limitations of serum therapy; with a summary of the results so far observed in the clinic.

T HE intelligent employment of specific immune serum in the treatment of pneumonia has abundant factual justification. The scientific background based on precise animal experimentation and the immunological study of artificial infection in animals and natural infection in man makes it possible to predict with a considerable degree of certainty the probable effect of antipneumococcic serum in controlling infection by Pneumococcus, and to understand some of the very obvious present limitations of specific serum therapy. The results at the bedside bear out the theoretical expectations.

The Rationale of Serum Therapy

The presence of virulent pneumococci in susceptible tissues stirs the latent physiological functions of the body to elaborate substances antagonistic to Pneumococcus. The same immunological defenses may be called into being by introducing into the sound animal body parts or the whole of artificially attenuated pneumococci.

The specific immune substances elaborated in response to the antigenic stimulus are extruded from the tissue cells into the circulating blood in which they may be identified and measured. These antibodies, through the medium of the serum in which they are present, may be transferred to the blood stream of man already beleaguered by pneumococci and, by combining with the cocci, ren-

der them susceptible to the attack of the natural protective forces of the body. The chapters on antigenicity (X), antibody formation (XI), and host response (XII) enumerate the several antigenic elements of the pneumococcal cell with their common or individual characters, trace the activity of these constituents in the animal economy, and describe the nature of the antagonistic substances thus created.

Problems and Limitations of Serum Therapy

The immunologist has succeeded in raising the antibody content of the serum of experimental animals to a level high above that engendered by spontaneous pneumococcal disease in man, but specific immune serum, even though it be rich in these protective principles, cannot convey to the recipient those less tangible properties acquired by the tissues as a result of antigenic stimulation. This failure constitutes one limitation of serum treatment.

The variety of serological types of Pneumococcus presents another complication, since the serum to be effective must correspond in type to the organism causing the infection to be treated. Antipneumococcic serum, therefore, does not have the broad immunological species-specificity of diphtheria or tetanus antitoxin; its affinities are strictly specific for type. Moreover, no matter how rich in type-specific antibodies a serum may be, when the mass of infecting pneumococci exceeds a given amount—all other conditions being equal—the serum is powerless to stop the multiplication, invasion, and subsequent malignant activities of the organisms within the infected host. The interaction of antibodies and pneumococci does not operate strictly according to the law of multiple proportions, and this relation between concentration of·specific antibody and living pneumococcal cells may determine the outcome of the infection. There are variations in this relation which are not yet understood. The ratio of amount of antibody to number of cocci is lower for Type I than for Type II. The utter

inadequacy of Type III immune horse serum in the treatment of lobar pneumonia caused by organisms of that serological type is still one of the riddles of pneumococcal research.

The substitution of immune rabbit serum for immune horse serum in the treatment of lobar pneumonia holds promising possibilities. From the experimental standpoint, antipneumococcic serum produced in rabbits confers a greater degree of protection on mice in proportion to the content of specifically precipitable protein than does antipneumococcic serum obtained from horses. In mice, small amounts of cholesterol, cephalin, or materials rich in these lipids block the protective action of antipneumococcic horse serum but are without effect in the case of rabbit serum. In massive infections in laboratory animals there are optimal amounts of antipneumococcic horse serum which must be used in order to obtain successful results. Larger amounts are less efficacious and still larger amounts may have no protective action whatever. The prozone effect is not observed with antipneumococcic rabbit serum.

As compared with horses, rabbits almost invariably respond to pneumococcal immunization with the production of serum of good titer. If serum treatment is to be carried out in all cases due to any of the thirty or more serological types, the production of these serums would be greatly simplified by employing small animals rather than horses. It is also probable that the cost of producing immune rabbit serum may be less than the cost of producing concentrated antipneumococcic horse serum. Preliminary clinical trials with antipneumococcic rabbit serum have been sufficiently encouraging to invite further study of this seemingly important advance in the serum treatment of lobar pneumonia.*

The details of the mechanism by which immune serum overcomes pneumococcal infections are only partly known. When the immune serum is type-specific and high in antibody content, the cocci are clumped and possibly to some degree removed from the circulation

* The foregoing statements are the conclusions of Horsfall, Goodner, and MacLeod.[659]

by the filtering action of the viscera. Antibody, by combining with somatic or capsular substance of homologous pneumococci, inhibits some of the vital activities of the cells and renders them more susceptible to ingestion by leucocytes. The completeness of the phagocytosis and the ability of the body to dispose of any remaining free cocci determine whether recovery or death is to take place.

There are other unexplained aspects of serum action. Serum may bear components antagonistic to its own antibodies, creating a restricted zone of effective action. The phenomenon is involved in the question of the optimal dosage of serum—a question influenced by the potency of the particular lot of serum at hand and the type and severity of the infection to be treated. Underlying these specific immunological considerations is the non-specific factor represented by the physical ability of the patient to cope with the invading pneumococci. Debilitation from any cause detracts from the active properties of the serum, but the matter is one largely of clinical interest. There are conceivably as yet unknown qualities of human blood or human tissue cells as well as of pneumococci which may either augment or decrease the curative action of specific immune serum.

The conditions stipulated by Neufeld and Haendel[992-3] in 1912 for the successful use of antipneumococcic serum have been subject to only slight modification in the intervening years. Based on the German authors' recommendations, the following main directions should guide the physician if serum is to be administered to greatest advantage:

1. The serological type of Pneumococcus infecting the patient should be determined with minimal delay, and the serum should correspond immunologically to the infecting organism.

2. The serum should be of known high potency and injected intravenously at the earliest possible moment after diagnosis is established. It is during the incipiency of the disease before pneumonic consolidation is advanced and before invasion of the vascular system is massive that the *Schwellenwert* of the serum is theoretically at its highest. Se-

rum administered in the late stages of the disease may be harmful to the patient.

3. The dosage of serum to be given will depend on the potency of the preparation, the type of organism involved, the severity of the infection, and the presence of complications such as pregnancy.

Reliable directions for testing the patient for hypersensitiveness to serum protein, for the technique used in the actual administration of serum, and the details of the many clinical attentions to be accorded the pneumonia sufferer are to be found in Lord and Heffron's *Lobar Pneumonia and Serum Therapy*.[827]

In the array of experiences already reported in the literature, the foregoing conditions presumably have not always been fulfilled. Serum of low potency or of heterologous type has been employed; the dosage has been sometimes insufficient and at other times excessive; injections have often been delayed beyond the most favorable time in the course of the disease; and it is conceivable that the bedside care has not always been ideal. Other derogatory conditions may obscure the true value of serum treatment. Nevertheless, the accumulated statistics taken as a whole, and especially those culled from selected series of cases in which serum therapy has been applied with proper regard for the many contributing circumstances, prove the value of antipneumococcic serum as a remedial agent in some of the infections caused by Pneumococcus.

In the above summary, only those data have been selected that bear evidence of the past and present status of serum therapy. The ensuing discussion is borrowed entire from Lord and Heffron's *Lobar Pneumonia and Serum Therapy*, Chapter X, Results of Serum Treatment. The numbers given to bibliographical references have been changed to correspond with those in the present volume.

The Results of Serum Therapy

For the continued and productive study of lobar pneumonia and the early, persistent, and successful application of serum therapy, we are largely indebted to Cole and his associates at the Hospital

of the Rockefeller Institute for Medical Research. Following the favorable report in 1913 by Cole and Dochez[267] on the treatment of Type I pneumococcus pneumonia, equally favorable reports from this source have from time to time been presented on an increasingly large number of cases. By 1929, Cole's[265] treated series numbered 371 with a case fatality rate of only 10.5 per cent. In addition to the low death rate in the treated group, such important supporting evidence as the cessation of spread, rapid appearance of protective antibodies in the blood, and the prevention or disappearance of blood invasion in consequence of specific therapy has been presented. In spite of these brilliant therapeutic results, the advantage of specific treatment was not generally recognized for some time. This was because the expected fatality rate in a large series of cases treated without serum had not been determined. The average fatality rate from a small early series of untreated Type I pneumonias was estimated at 25 to 30 per cent, and, convinced of the effectiveness of antiserum, Cole did not feel justified in withholding the treatment in a control series.

These fatality rates of cases treated with and without specific therapy have since been confirmed, but only after a long period of uncertainty on the part of the general medical profession. Unfortunately others had less striking success than Cole, and Locke's[820] report in 1923 of a death rate of 17.2 per cent in 145 serum treated cases (only 12 of which were treated in the first three days) and practically the same death rate, 16.9 per cent, in 71 untreated cases raised serious doubts in the minds of many regarding the merit of the procedure. These doubts were resolved by further determination of the comparative results with and without antiserum in contemporaneous series and an analysis of the treated group according to the time when specific treatment was begun. Among others, Lord; Cecil and Sutliff; Park, Bullowa and Rosenblüth; Finland; Cecil and Plummer; and Sutliff and Finland showed that successful specific treatment depends largely on its application early in the course of the disease.

For several hours after the administration of the initial doses

of antiserum, no change in the patient is likely to be observed, but within eight to twenty-four hours after the first dose there is usually a marked improvement. This favorable response is likely to be more pronounced in the case of Type I than in the case of Type II infections and when patients are treated early. The patient becomes clearer mentally and begins to take an interest in his surroundings. Cyanosis and dyspnea may be completely relieved. In a large majority of the cases the temperature drops two or three degrees or more and does not again reach its former level (Figure 1). At the same time, though less rapidly, there is a fall in the rate of the pulse and respiration. In patients not treated with serum, Sutliff and Finland[1362] found that a much smaller proportion show symptomatic improvement and a sharp drop in temperature during the course of the disease. Following this initial fall in the temperature of serum treated cases, there may be a continued low-grade fever and lesser complaints for several days.

Studies by Sutliff and Finland on a series of serum treated cases compared with others not so treated indicate that the average duration of the more acute symptoms measured from onset to a persistent lowering of the temperature is shortened by about thirty hours in the serum treated group. The average duration of the low-grade fever following the relief of the more acute symptoms, however, is about the same in both groups. The disease runs a milder course following serum therapy.

An even more marked shortening of the acute illness in the treated as compared with control cases is reported in Great Britain by the Therapeutic Trials Committee of the Medical Research Council.[887] They find, on the average, that crisis occurred two and one-half days earlier in the treated than in the control Type I cases, and two days earlier in the treated than in the control Type II cases. In other instances where the progress of cases was analyzed in a different way, they note that, compared with the control cases, from four to seven times as many treated Type I cases show defervescence on or before the fifth day of illness, and from three to six times as many Type II cases.

FIGURE 1

COMPOSITE TEMPERATURE CHART OF 320 RECOVERED TYPE I
CASES WHOSE TREATMENT WITH CONCENTRATED SERUM
WAS BEGUN WITHIN 96 HOURS OF ONSET
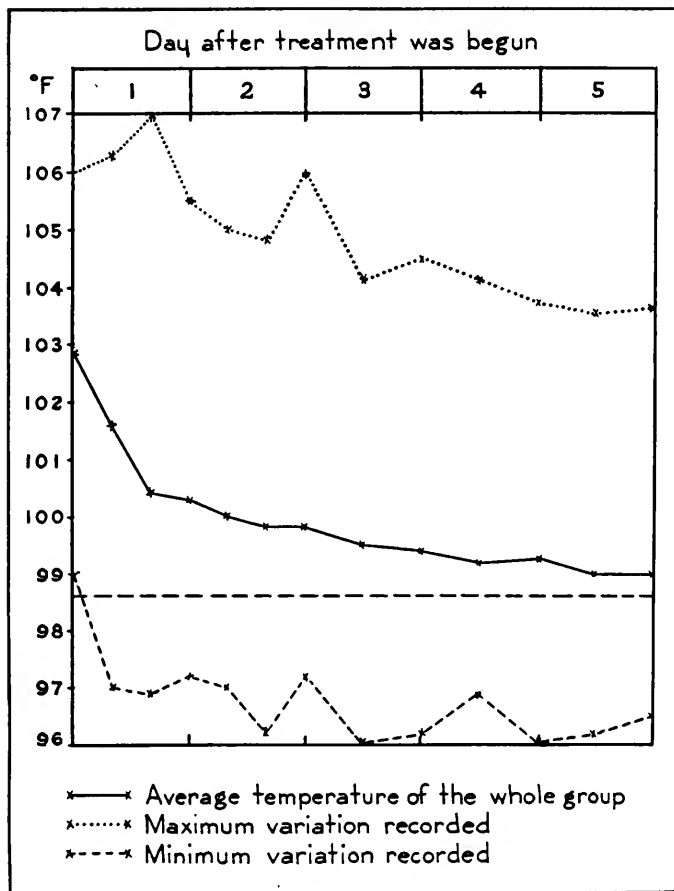
Temperatures are shown at eight-hour intervals for the first forty-eight hours after the beginning of treatment and then twice daily.

For 27 of the 320 cases treatment was begun on the first day of illness; for 101 on the second day; for 120 on the third day; for 72 on the fourth day.

Cases are taken from the Massachusetts Pneumonia Study. The remaining 128 recovered cases are not included as their temperature charts are not available or are inadequate for the purpose.

Specific serum therapy has a marked effect in preventing blood invasion, and blood cultures previously positive usually become negative within a few to twenty-four hours or more. The area of lung involvement with few exceptions ceases to spread. If extension occurs, it takes place slowly. By contrast, extension is frequent in those not treated with antiserum. Resolution appears to be unrelated to immunity factors and is not obviously affected by specific treatment.

Antiserum appears to have little or no effect in reducing such complications as endocarditis and meningitis. Observations in Great Britain[887] suggest that the incidence of empyema may be reduced.

The crucial test of the value of antiserum is its effect on the case fatality rate. The expected case fatality rate without specific therapy for Type I infections may be estimated from a large series of civilian cases, collected from the literature by Heffron, at about 25 per cent. Of 1,614 cases occurring in the United States and Canada, 403 or 25.0 per cent died (Figure 2). In Great Britain the fatality rate of Type I cases is somewhat lower than this.

FIGURE 2

TYPE I: COMPARISON OF DEATH RATES OF CASES WHOSE SERUM
TREATMENT WAS BEGUN WITHIN NINETY-SIX HOURS OF
ONSET AND OF CASES NOT GIVEN SERUM

Treated with serum* ▦▦▦▦▦ 11.1%
504 cases, 56 deaths

Not treated with serum† ▦▦▦▦▦▦▦▦▦ 25.0%
1,614 cases, 403 deaths
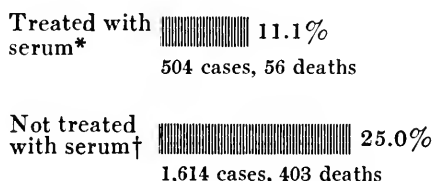
* Massachusetts Pneumonia Study cases.
† Cases collected from the literature.

With these figures for comparison, Heffron finds that of 2,458

Type I serum treated* cases in the literature, 384 or 15.6 per cent died, indicating the saving of many lives. The expected death rate of 25 per cent can be still further reduced, as shown by the results in the Massachusetts cases, when the treatment is begun within the first four days (ninety-six hours) of the onset. Of 504 cases thus treated with concentrated serum, only 56 or 11.1 per cent died (Figure 2). The importance of time is further emphasized by the figures shown in the accompanying table. Of 377 cases of Type I pneumonia treated within the first three days, only 32 or 8.5 per cent died. The death rate rises abruptly by the fourth day. Of the 127 cases treated on this day, 24 died, or 18.9 per cent.

TABLE I

TYPE I CASES TREATED WITH SERUM. NUMBER AND PERCENTAGE OF DEATHS BY DAY ON WHICH SERUM TREATMENT WAS BEGUN

Serum treatment begun	Number of cases	Deaths	
		Number	*Per cent*
In first three days	377	32	8.5
On fourth day	127	24	18.9
Total	*504*	*56*	*11.1*

NOTE: Cases were taken from the Massachusetts Pneumonia Study.

The fatality rate is, however, diminished when treatment is begun still later in the illness, but less strikingly than in the earlier period.

Similarly, Heffron has collected from the literature 992 Type II cases treated without serum in the United States and Canada, with 407 deaths, a fatality rate of 41.0 per cent (Figure 3). By contrast, he finds 670 antiserum treated* Type II infections in the literature with 205 deaths, or 30.6 per cent. Of 136 such cases in the Massachusetts series, treated with concentrated serum within four days (ninety-six hours) of onset, 37 died, a fatality rate of 27.2 per cent (Figure 3).

* With unconcentrated or concentrated serum in the United States or Canada.

FIGURE 3

TYPE II: COMPARISON OF DEATH RATES OF CASES WHOSE SERUM
TREATMENT WAS BEGUN WITHIN NINETY-SIX HOURS OF
ONSET AND OF CASES NOT GIVEN SERUM

Treated with serum* ||||||||||||||||||||||||||||||||||| 27.2%
136 cases, 37 deaths

Not treated with serum† ||||||||||||||||||||||||||||||||||||||||||||||| 41.0%
992 cases, 407 deaths
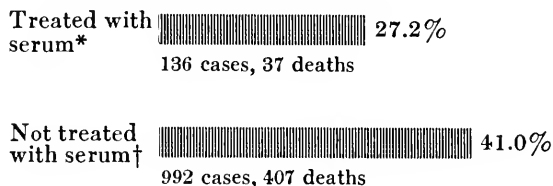
* Massachusetts Pneumonia Study cases.
† Cases collected from the literature.

The lowering of the death rate in the serum treated Type I and II cases in Massachusetts is of special importance in indicating that the method can be satisfactorily used by practicing physicians outside of large hospitals. Over one half were treated by physicians in general practice in the home or in small hospitals in the state.

FIGURE 4

TYPE I CASES TREATED WITH SERUM AND NOT TREATED
WITH SERUM. NUMBER AND PERCENTAGE
OF DEATHS BY AGE GROUP

10–49 years of age   50 years of age and over

Treated with serum* |||| 8.3% ||||||||||| 30.8%
432 cases, 36 deaths   65 cases, 20 deaths

Not treated with serum ||||||| 22.0% |||||||||||||||||| 60.7%
141 cases, 31 deaths   28 cases, 17 deaths
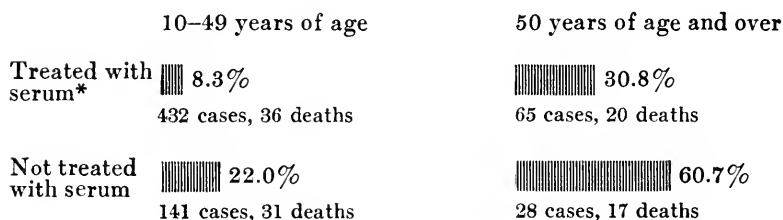
* Seven cases were not included as the age was not known.

NOTE: Cases were taken from the Massachusetts Pneumonia Study.

Advancing years is, in general, one of the most important factors in unfavorably modifying the outlook with lobar pneumonia. As shown in the accompanying figure (Figure 4), the case fatality rate is 22 per cent without and 8.3 per cent with specific treatment in patients from ten to fifty years of age, and 60.7 per cent without and 30.8 per cent with such treatment in patients fifty years of age and over. Analysis of the data with respect to the age of patients with and without specific therapy indicates that the favorable results of treatment cannot be ascribed to a difference in the ages of patients in the two groups.

As previously noted, bacteriemia occurs in about a quarter of Type I infections and in about a third of Type II infections. As shown in the accompanying figure (Figure 5), the occurrence of blood invasion in patients treated without antiserum greatly in-

FIGURE 5

TYPE I AND TYPE II CASES WITH AND WITHOUT BACTERIEMIA
NOT TREATED WITH SERUM. NUMBER AND
PERCENTAGE OF DEATHS

Type I	Type II
Cultures negative ▨ 18.1%	▨ 13.3%
254 cases, 46 deaths	315 cases, 42 deaths
Cultures positive ▨ 64.6%	▨ 76.2%
155 cases, 100 deaths	227 cases, 173 deaths

NOTE: Figures were collected from the literature. No cases of
the Massachusetts Pneumonia Study were included.

creases the death rate. Thus the fatality rate in Type I cases with negative cultures is 18.1 per cent and with positive cultures 64.6 per cent. The situation is much the same with Type II infections, the death rate rising from 13.3 per cent with negative to 76.2 per

cent with positive cultures. With bacteriemia, the death rate is thus about three to five times as great. The larger the number of organisms per cubic centimeter of blood, the more unfavorable the outlook.

As shown in Figure 6, specific treatment lowers the death rate in bacteriemic cases, in Type I infections from 64.6 to 31 per cent

FIGURE 6

TYPE I AND TYPE II CASES WITH BACTERIEMIA TREATED
WITH SERUM AND NOT TREATED WITH SERUM.
NUMBER AND PERCENTAGE OF DEATHS

	Type I	Type II
Treated with serum	▥▥▥▥ 31.0%	▥▥▥▥▥ 52.9%
	271 cases, 84 deaths	68 cases, 36 deaths
Not treated with serum	▥▥▥▥▥▥▥ 64.6%	▥▥▥▥▥▥▥▥ 76.2%
	155 cases, 100 deaths	227 cases, 173 deaths

NOTE: Cases were collected from the literature. No cases from the Massachusetts Pneumonia Study were included.

and in Type II from 76.2 to 52.9 per cent. As shown in Figure 7, when serum treatment is given within the first four days (ninety-six hours) in Type I infections, the fatality rate is reduced to 26.2 per cent and in Type II to 54.2 per cent, the corresponding fatality rate with early specific treatment and negative cultures being respectively 5.2 per cent and 16.7 per cent.

With respect to the possibility that selection may have modified the case fatality rates of reported series, it may be assumed that exclusion of patients sensitive to horse serum from treated groups and their inclusion in control groups did not of itself modify the death rate. When the age has been averaged, it has been found to be approximately the same in the treated and the

FIGURE 7

TYPE I AND TYPE II CASES WITH AND WITHOUT BAC-
TERIEMIA TREATED WITH SERUM. NUMBER
AND PERCENTAGE OF DEATHS

Type I   Type II

Cultures ||| 5.2% ||||||| 16.7%
negative
    191 cases, 10 deaths   54 cases, 9 deaths

Cultures |||||||||| 26.2% |||||||||||||||||||| 54.2%
positive
    61 cases, 16 deaths   24 cases, 13 deaths

NOTE: Cases were taken from the Massachusetts Pneumonia Study.

untreated groups. The inauguration of treatment within the first four days makes it improbable that any important number of cases were excluded because in extremis from pneumococcus infection.

Certain avoidable causes of failure in the application of specific therapy may be briefly reviewed. Chief among these is the delay before beginning treatment. Second in importance is the lapse of time, previously often unavoidable, required for the determination of the type of infection and now largely obviated by the use of the Neufeld method. A third factor is the acceptance of other than Type I and Type II pneumococci in the sputum as the etiologic agent without repetition of the typing and a possible consequent error of about 10 per cent. Other causes may be the use of insufficient antiserum, low potency, or too long intervals between doses and failure to recognize the importance of administering more than the usual amount of antibody in severe cases and with pregnancy, during the post-partum period, and with bacteriemia.

Among unavoidable causes of failure, the presence of complications, and especially empyema, is not infrequent. Mixed infection with more than one type of pneumococcus, the streptococcus, or

other organism may be present. Lastly, the infection may be so severe that specific treatment is ineffectual, or the infection may be overcome but the patient, exhausted by the struggle, succumbs.

Summary

A notable beginning has been made, but it is after all only a beginning. With the development of more potent and refined serum, possibly from an animal other than the horse, and a better knowledge of dosage, with a wider appreciation of the urgency of early diagnosis and prompt administration of serum, and with greater discrimination in the use of this biological agent, the doubts which linger in many a medical mind should be largely dissipated and specific serum treatment should advance to an even more important place than that which it now holds in the treatment of pneumonia.

UNSOLVED PROBLEMS

IMPRESSIVE as is the progress that has been made in Pneumococ-
cus research, the subject holds almost endless possibilities for
further investigation. Some of the metabolic activities of Pneumo-
coccus, the processes operating in dissociation and type transfor-
mation, the exact chemical composition and precise antigenic
properties of the pneumococcal cell, these are subjects, among
many others, calling for continued study.

It is probable that the heights which may be achieved in the pro-
duction of artificial immunity to Pneumococcus in man and in
serum-producing animals have not been reached. Antipneumococ-
cic serum, at best, leaves much to be desired in the way of purity
and potency. There are many inviting leads and, with the applica-
tion of rapidly developing chemical and serological methods, the
years to come should be even more fruitful than those of the past.

TOXINS

The idea persists, in spite of many disappointing failures, that
some of the symptoms incident to pneumococcal infection, espe-
cially those of lobar pneumonia, are caused by a soluble toxin.
The idea is a reasonable one but convincing evidence to support it
has never been presented. Among the varied activities of the pneu-
mococcal cell a substance of this nature may be produced. It may
exist in nascent or evanescent form or be present in such minute
quantities as to escape detection. Conditions that exist in the
animal body cannot be reproduced in full in lifeless media and
therefore the environment supplied by artificial culture is quite
different from that encountered by microorganisms in living tis-
sues. There is the well-known influence of specific substrates on the
biochemical activities and physiological processes of bacteria in

revealing potential or latent functions not active or even suspected when the same cells are grown in ordinary media. Toxins as such may not be elaborated by pneumococci themselves in an artificial substrate but may possibly be a secondary product of the interaction between the bacteria and the cells of the host. The question is by no means closed.

SEROLOGICAL TYPES

The present division of pneumococci into thirty-two distinct serological types may not be all-inclusive. Further study of strains at present considered as unclassifiable and continued search of fresh material will undoubtedly add other specific types to the list, although the operation of the law of diminishing returns is inevitable.

There is increasing need for a central source of type-specific diagnostic serum to ensure the accuracy and uniformity of type-determination throughout this and other countries. The problem is largely an administrative one but the requirements come from bacteriologists, immunologists, and epidemiologists. The movement to establish such a center is to be strongly encouraged, and might well be included in the deliberations of the Health Organization of the League of Nations.

DISSOCIATION

A reconciliation of the many descriptions of pneumococcal variants is greatly to be desired. Modifications A to Zed, the bizarre forms, the mucoid, smooth, and rough variants should be sorted into a uniform, descriptive grouping, and the designations, if possible, should be made to conform to those of similar variant forms of other bacterial species.

The unknown principle in the virulent pneumococcal cell which has the remarkable ability not only to restore a degraded form to its original condition of type-specificity, but which also can actu-

ally transform the degraded variant into a member of a different serological type, invites further study.

Does dissociation of pneumococci proceed in the animal body? At present the answer is, Probably not; but the manner in which the many types of pneumococci have arisen may some day be discovered to be related to the influences that cause variation, reversion, and transformation of type.

The supposition that the close relation apparently existing between pneumococci and streptococci denotes a divergent evolution from a common progenitor does not bear analysis in a genetic sense. Bacteria are unicellular organisms reproducing by the elementary process of fission and it is far more likely that environmental influences induce or result in more or less transient physiological or protoplasmic changes that are wholly or in part reversible when the cells grow under other conditions. The recognition of the stability of biological types or species, therefore, leads rather to continued study of the cultural factors that bring about temporary modifications in bacterial characters.

CHEMICAL CONSTITUENTS OF PNEUMOCOCCI

The knowledge gained in the last score of years concerning the chemical nature of the components of the pneumococcal cell furnishes abundant material for a broad program of future biochemical and immunological studies. How closely do the proteins and polysaccharides thus far isolated correspond in molecular configuration to the parent substances as they exist in the living, intact cell? What are the relationships between chemical constitution and biological specificity? What is the exact grouping and what are the linkages of radicals that determine for a strain its type-identity? Are there actually antigenic and haptenic groups in some of the specific carbohydrates, or are antibody stimulation and antibody combination only quantitative manifestations of the same phenomenon? How can one account for the partial sero-

logical similarities or differences between the polysaccharides of the present pneumococcal types and the polysaccharides of other bacteria and non-living substances?

What is the importance of the chemical configuration of the molecule as a whole and the role of determinant groups in orienting the specificity of the immune response in animals? What is the nature of the chemical processes in the bodies of animals of different species which deal with one and the same antigenic substance in such diverse ways after it reaches the tissues? For example, it is possible that a complex antigen may be broken down to non-antigenic form or to a simple hapten-like substance, so modified by conjugation or synthesis within the tissues that it acquires new antigenic properties—the result depending not only upon the chemical nature of the substance introduced and the route of its administration, but also upon the chemical changes brought about in the particular tissue or animal species tested.

These are some of the problems that confront the chemist and the immunologist. Perhaps the isolation of specific polysaccharides from pneumococci of the remaining unstudied types, from unrelated organisms, and from other sources, and an investigation into the more minute details of the molecular mosaic of these higher carbohydrates, may provide clues to the solution of some of these problems.

The fact that bacterial enzymes capable of decomposing capsular polysaccharides of the different pneumococcal types have more avid and more discriminating selective affinities for capsular polysaccharides than homologous antibodies have for specific antigens, may lead to the clarification of some of the questions of chemoimmunological specificity.

VIRULENCE

Why a strain of Pneumococcus should be virulent for one species of animal and avirulent for another is a question that awaits answer, and the question is not a simple one. There are the ele-

ments in pneumococci that endow them with invasive powers and, among these elements, the type-specific capsular polysaccharide may be the principal determinant. The reasons for the differences in the virulence characteristic of pneumococcal types, of individual members within the types, and of variant forms may be linked with capsule formation, but that is only one element of this vital character. The unusual virulence of Type III pneumococci for man as compared with animals, and the invasiveness of some strains for rabbits and the harmlessness of others are striking examples of the problems to be studied. There are also marked differences in host response among various animal species and among animals within the species, and here the matter is largely beyond our ken.

Are the capsular polysaccharides toxic in themselves, or do they combine with tissue elements to produce poisonous effects or, as is now being discovered in the case of the polysaccharides of certain bacteria of the enteric group, do these carbohydrates, possibly linked with lipids, exist in a still more complex form than any yet isolated?

METHODS FOR THE PRODUCTION OF ACTIVE IMMUNITY

Procedures for the creation of active immunity in animals and in man are in a state of development. The ideal immunizing antigen and the most effective scheme for administering the antigen are yet to be found. The polysaccharides of Pneumococcus either alone or in a form to be discovered or invented may prove to be the sought-for agent. The possibilities are manifold.

Is it the protective antibody which must especially be striven for or are other immune substances also required to build resistance to infection? What part—if any—do heterophile antibodies contribute and, more particularly than we now know, what part do the somatic cells play in protecting the infected host, and do other physiological factors share in creating the immune state?

The serum of animals immunized by the injection of pneumococcal materials, besides bearing specific antibodies, contains sub-

stances antagonistic to the activity of the antibodies and limiting
the protective action of the serum. By what means can these in-
hibitory substances be neutralized or eliminated?

Are the zonal effects observed in serological reactions and in
protective experiments ascribable wholly to the capsular polysac-
charide of Pneumococcus or may they be, in part at least, pro-
duced by serum lipids? To what extent are these effects dependent
upon the quantitative relations existing at the time between anti-
gen and antibody and the solubility of the immune complex so
formed?

CHEMOTHERAPY

A suitable and harmless drug with high selective affinity for the
protoplasm or capsular substance of Pneumococcus is wanting.
The field of chemotherapy in pneumococcal infections, promising
as it is, abounds in difficulties. The narrow limits between poison-
ous, untoward effects and therapeutic action in the animal body,
the selective affinity of the agents for somatic protein or capsular
carbohydrate of bacteria, and the action of the drug in inhibiting
some essential physiological function, impairing the metabolic
processes, or bringing about morphological changes in both bac-
terial and body cells, are problems that should entice rather than
discourage the investigator.

At present, cinchona would appear to offer a promising source
from which to prepare agents capable of specific pneumococcidal
action. In the myriad of compounds now being synthesized in the
chemical laboratory a key substance may be found.

IMMUNOLOGICAL RESPONSE

The comparative response of animals of different species to the
introduction of pneumococcal antigens by diverse parenteral
routes perplexes the immunologist. Antigen injected into the skin
of the rabbit results in the appearance of species-specific but not
of type-specific antibodies in the serum; antigen injected into a

vein of the same animal evokes the formation of antibodies of strict type-specificity homologous with the type of antigen employed. If the nature of the tissue reaction in each case could be discovered, our conception of immunological processes would acquire further definition.

The local fixation of bacteria at the site of injection into the skin of rabbits may allow opportunity for the action of tissue enzymes to inactivate or dissociate capsular antigen of the bacterial cell, leading to or accounting for the diminution or loss of type-specific response. The assumption is susceptible of proof.

ANTIBODIES AND ANIMAL SPECIES

The immunological differences between antipneumococcic serum produced in rabbits and that produced in horses have been mentioned in earlier parts of the text. There may be, of course, quantitative differences in the antibody content of the two serums; the antibody may exist in one fraction of one and in another fraction of the other serum, and its affinity for antigen may be modified by its particular conjugation with some serum component. We are now learning that the molecular size of the antibody or the physical state in which antibody exists in immune serum may account for these differences in serological behavior.

SKIN REACTIONS

The history of the phenomena to be observed following the intradermal injection of pneumococcal materials is incomplete and confused. The diversity of antigenic preparations employed, and the variety of inflammatory reactions induced in the cutaneous tissues and classed as positive, largely account for the confusion. The antigens comprising somatic protein and carbohydrate and capsular polysaccharide should be prepared in accordance with the most advanced technique and should be selected as representative of the chemical and immunological individuality interpreted in terms of the specific reactions of the organisms as a whole. The

local effects following the introduction of pneumococcal antigens into the skin should be more clearly defined and classified and all interpretations should be based on such definition and classification.

The problems involved in the several kinds of reactions elicited, their relation to the clinical course of infection, and their interpretation in respect to preexisting or newly developing states of hypersensitiveness and immunity are still to be studied and solved. Recent studies of the somatic C polysaccharide suggest that certain forms of reactivity, both serological and cutaneous, are not specific in an immunological sense, but are rather an expression of chemical changes in the blood associated with the acute stage of active infection.

THE NATURE OF PNEUMOCOCCAL ANTIBODY

The behavior of antibodies of immune serum in the presence of protein precipitants and the molecular size of the immune substances as determined by ultracentrifugation and ultrafiltration lead to the tentative assumption that these antibodies represent modified forms of serum proteins, having in general the chemical properties of globulins. The isolation by advanced methods of antibody or antibodies in purer form than that in which they have been previously obtained promises to be a desirable point of departure for further inquiry into the chemical nature of antibodies, the elucidation of the mechanism of immunological reaction, and for the development of a more efficient therapeutic serum.

CONCENTRATION OF ANTIPNEUMOCOCCIC SERUM

Methods for concentrating and refining antipneumococcic serum have undergone notable improvement, but it cannot be claimed that the agent approaches the perfection of diphtheria and tetanus antitoxin. Raw serum of greater potency is the first requisite, and then there must be devised chemical procedures which will make it possible to separate the desired antibody or antibodies

from the immunologically inert and frequently undesirable com-
ponents of serum without at the same time denaturing or unduly
destroying the wanted immune substances. The economical concen-
tration of the unimpaired curative principles of serum in small
bulk in a solution of bland physiological action is the goal to be
striven for. From a purely practical standpoint, if antipneumococ-
cic serum is to render its greatest service, aside from an appre-
ciable lowering of the cost of the product, the elimination of super-
fluous protein and of antagonistic or chill-producing substances
constitutes a step of more immediate need.

POTENCY TESTS ON THERAPEUTIC SERUM

The early proximal or presumptive tests employed for estimat-
ing the curative value of antipneumococcic serums have gradually
been supplanted by methods that ensure a greater degree of accu-
racy in measuring the antibody content of the product. Great as
has been the effort expended in devising a technique that will at the
same time be simple and exact and afford a reliable index of the
curative value of the product, no method so far devised can be
looked upon as ideal. The principles involved are discouragingly
more complex than those met in the titration of antitoxic serums.
The direction that further endeavors should take is toward econ-
omy of means and time and a closer correlation between antibody
titer and therapeutic strength. It is now generally taken for
granted that precipitin and protective titer are the only factors
to be considered, but the possible importance of allied antibodies
should not be overlooked.

The fairly successful production of specific serum for Type I
infections encourages efforts to prepare a product of enhanced
potency for Type II pneumonia and to attack from new angles the
baffling puzzle presented by Type III Pneumococcus. The devel-
opment of monovalent immune rabbit serums for these types and
for the types of relatively low incidence holds promising possi-
bilities.

SUMMARY

There are, of course, many knotty problems regarding the epidemiology of lobar pneumonia ; the nature of contributory factors and altered physiological states associated with the occurrence and onset of the disease ; the carrier state ; the mode of dissemination of the infectious agent ; the portal of entrance and pathways of infection in the body ; and the mechanism of crisis and recovery. These problems, while involved in the larger domain of the biology of Pneumococcus, are of less immediate interest to the bacteriologist and immunologist than to the epidemiologist, the physiologist, the pathologist, and the clinician.

Opportunities for inquiries into the ways of Pneumococcus are by no means exhausted. The prosecution of future studies on the life activities of Pneumococcus cannot fail to bring new gold to the abundant store of solid bullion already accumulated. Those workers who are not as yet engaged in this field of biological research, as well as those who are already devoting their energies to solving the riddles presented by this amazing bacterial cell, may, it is hoped, gain some enlightenment and possibly a few prospects in the foregoing reexamination and appraisal of our gains.

SPECIAL METHODS USED IN THE STUDY OF PNEUMOCOCCUS AND IN THE PREPARATION OF ANTIPNEUMOCOCCIC SERUM

I. Media

PNEUMOCOCCUS BROTH FOR ALL GENERAL PURPOSES (Rockefeller Institute. Courtesy of Miss Dorothy Sloan)

Beef heart	1 pound
Peptone (Witte or Pfanstiehl)	10 grams
Sodium chloride	5 grams
Tap water	1 liter

The beef hearts are obtained directly from the slaughter-house and after immediately removing all fat, the muscle is ground fine. For every 10 liters of water add 10 lb. of minced heart muscle and 500 cc. extra of water. Stir and skim off fat rising to the surface. Repeat stirring and skimming ten times. Place in refrigerator overnight, heat to 85° for one-half hour, then filter through Prat-Dumas No. 50 or other coarse filter paper. Add peptone and salt, reheat to boiling and add sufficient N sodium hydroxide to bring the reaction of the medium to pH 8.2. Boil for 5 minutes and filter immediately through coarse paper. Sterilize in the Arnold sterilizer for one hour on each of 3 successive days. The final reaction should be pH 7.8.

For the production of massive amounts of pneumococci, the medium in which the Pfanstiehl peptone is used is inoculated with 100 to 150 cc. of an 8-hour culture in plain broth for every 3,000 cc. of medium. The maximal growth will have occurred in 8 to 10 hours under these circumstances, but the yield of organisms is greatly increased by the addition of 0.10 per cent dextrose after 8 hours' incubation. Following the addition of dextrose, incubation is carried out for one to one and one-half hour; longer incubation will render the medium too acid and rapid autolysis will result. The resulting growth is immediately collected by means of a specially constructed Sharples centrifuge.

NUTRIENT BROTH FOR MASS CULTURES FOR THE PRODUCTION OF SOLUBLE SPECIFIC SUBSTANCES (Courtesy of Dr. F. D. Hager)

a. *Preparation of infusion.* Fresh horse meat is ground and mixed

with aliquot parts of chipped ice and cold tap water. The mixture is stored in earthenware jars in the cold room for 3 to 5 days and is stirred at intervals. After most of the ice has melted, the mixture is transferred to aluminum kettles, heated to boiling, strained through cheesecloth, and the cake of coagulated meat pressed as nearly dry as possible in a meat press. The strained liquid part of the infusion is collected in 5-gallon Pyrex carboys and autoclaved at 120° for 45 minutes. Air is then forced into the autoclave to keep the pressure at 15 to 20 pounds until the temperature falls to about 80° or 90°. The crude, sterilized extract is stored in the cold room.

b. *Preparation of medium.* The crude infusion is filtered in the cold room through soft filter paper. It is siphoned from the carboy to avoid disturbing the layer of partly solidified fat which collects on the surface. Unless the infusion is to be used for producing Type III SSS, it is best to remove the glycogen as follows:

The infusion is evaporated to about one-tenth its original volume, and the glycogen precipitated by the addition of two volumes of 95 per cent alcohol to one of infusion. The glycogen is separated by centrifugation. The glycogen-free infusion is then distilled under reduced pressure in a water-bath to free it from alcohol.

The glycogen-free, alcohol-free infusion is then made up to its original volume with tap water, and to it is added peptone (Witte or Pfanstiehl) to a concentration of 1 per cent. The solution is filtered through soft paper into a 5-gallon Pyrex carboy and autoclaved as described above.

The infusion-peptone solution may be diluted with an equal volume of tap water before autoclaving and use. This step results in a higher yield of polysaccharide per volume of infusion, but requires the handling of larger amounts of fluid. Prior to inoculation, the medium is stored in the incubator room for a sufficient time in order that it may reach the proper temperature.

A 30 to 40 per cent solution of glucose is added to the medium in amounts to give a final concentration of 1 per cent. The glucose is sterilized separately to prevent the formation of colored products which are difficult to separate from the capsular polysaccharide.

The alkali solution used in neutralizing the acid formed during growth is sterilized in the Arnold sterilizer for 30 minutes in calibrated Pyrex flasks fitted with a stopper bearing an air filter and a glass tube which reaches to the bottom of the flask. The other end of the tube is fastened to a rubber tube of sufficient length to reach from the

flask of alkali solution to the stopper of the carboy of medium. When half-strength medium is used or when the reaction is to be kept acid, 5 per cent NaOH is preferred; otherwise 10 per cent NaOH is more convenient.

c. *Growing the pneumococci.* There is placed in the carboy of medium a stopper which carries: (1) A mechanical stirrer of the usual sealed type; cresol solution is used as the sealing liquid. (2) An inlet tube to which the rubber tube from the flask of alkali solution can be attached. (3) An inlet tube through which can be added the glucose solution, defibrinated blood, and culture. (4) A sampling tube through which samples may be removed from the carboy without undue risk of contamination. The stirrer, stopper, and tubing are sterilized separately.

To the medium are added the glucose solution and about 50 cc. of defibrinated blood. The carboy is then placed on a stand so arranged that the stirrer can be driven by a motor and that the flask of alkali solution can be connected with the carboy. Alkali solution is then added to the medium in an amount sufficient to adjust the reaction to a pH of 7.0 or of 8.0 to 8.5 depending upon whether acetyl SSS or the ordinary SSS is to be made. The medium should be thoroughly stirred when the alkali is added.

Very late in the day about 5.0 cc. of an 8-hour culture of Pneumococcus is added to each carboy, the mixture stirred a few minutes, and the organisms allowed to grow overnight. By morning the pH will have fallen to about 4.5. The reaction is then readjusted to pH 7.0 or 8.0 to 8.5; the medium requires readjustment the same evening and both morning and evening of the second full day of growth, after which time adjustment of the reaction once a day will suffice. In the early stages of growth, a pH of 8.8 will not kill the culture. Appreciable acid production ceases after about a week.

The cultures are kept for about two weeks before starting preparation of capsular polysaccharide. The methods of Heidelberger, Avery, and their colleagues are followed in purifying the substance.

BLOOD BROTH

Plain broth or hormone medium to which defibrinated rabbit, horse, or human blood is added to 0.05˙ to 5 per cent concentration when the medium is cool. Rabbit blood is preferable.

INULIN SERUM WATER (Rockefeller Monograph No. 7[36])

For the determination of inulin fermentation by Pneumococcus, the

following medium devised by Hiss is used, a positive reaction being in-
dicated by the production of acid and the coagulation of serum protein.
Clear beef serum is added to 2 or 3 volumes of distilled water. Heat the
mixture for 15 minutes in an Arnold sterilizer at 100° to destroy fer-
ments present in the serum. Add 5 per cent aqueous litmus solution to
a concentration of 1 per cent or an amount sufficient to give a deep blue
color. Add inulin (C.P.) to the serum water to a concentration of 1 per
cent. The inulin solution may best be sterilized by autoclaving at 15
pounds pressure for 15 minutes. Sterilize the inulin serum water by the
fractional method at 100°.

AUTHOR's NOTE: An alcoholic solution of bromcresol purple is preferable to
the litmus solution as an indicator.

II. Isolation of Pneumococcus

MOUSE METHOD (Rockefeller Monograph No. 7[36])

a. *Mouse inoculation.* A small portion of the sputum, about the size
of a bean, is selected and washed through three or four changes of ster-
ile salt solution in sterile Esmarch or Petri dishes to remove surface
contaminations. When the sputum is too friable or when the specimen is
relatively free from secondary organisms, this washing process may be
omitted. In either event, the kernel of sputum selected is transferred to
a sterile mortar, ground up, and emulsified with about 1.0 cubic centi-
meter of sterile bouillon or salt solution, added drop by drop, until a
homogeneous emulsion is obtained that will readily pass through the
needle of a small syringe. With a sterile syringe, 0.5 to 1.0 cc. of this
emulsion is inoculated intraperitoneally into a white mouse. Pneumococ-
cus grows rapidly in the mouse peritoneum, while the majority of other
organisms rapidly die off, with the exception of Friedländer's bacillus,
Bacillus influenzae, and occasionally *Micrococcus catarrhalis,* Staphylo-
coccus, and Streptococcus. Pneumococcal invasion of the blood stream
also occurs early. *Bacillus influenzae,* if present, likewise invades the
blood stream; other organisms, as a rule, do not. The time elapsing be-
fore there is sufficient growth of Pneumococcus in the mouse peritoneum
for the satisfactory determination of type varies with the individual
case depending upon the abundance of pneumococci in the specimen of
sputum and the virulence and invasiveness of the strain present. It may
be from 5 to 24 hours, averaging 6 to 8 hours with the parasitic fixed
Types I, II, and III. As soon as the injected mouse appears sick, a
drop of peritoneal exudate is removed by means of peritoneal puncture

with a sterile capillary pipette, spread on a slide, stained by Gram's method, and examined microscopically to determine whether there is an abundant growth of Pneumococcus present. If there is an abundant growth of Pneumococcus alone, the mouse is killed and the determination of type carried out. If the growth is only moderate, or if other organisms are present in any quantity, additional time must be allowed until subsequent examination of the peritoneal exudate shows an abundant growth of Pneumococcus. It should be emphasized that undue haste in killing the mouse is time lost in the end.

b. *Mouse necropsy.* As soon as the mouse is killed or dies, the peritoneal cavity is opened under sterile precautions and cultures are made from the exudate in plain broth and on one-half of a blood-agar plate. Films are made and stained for microscopic examination by Gram's stain and Hiss's capsule stain. The peritoneal exudate is then washed out by means of a sterile glass pipette with 4 to 5 cc. of sterile salt solution, the washings being placed in a centrifuge tube. Cultures are then made from the heart's blood in plain broth and on the other half of the blood-agar plate. The essential steps in the procedures given above are shown in the accompanying Chart 1.

PLATING METHODS (Zinsser and Bayne-Jones[1579])

Although the methods used in preparing and using blood agar vary, the following illustrate accepted procedures:

a. *Preparation of blood agar*

(1) Melt 100 cc. of meat infusion or Douglas' agar (pH 7.4 to 7.8) in a flask.

(2) Allow to cool to 45° to 50°.

(3) Add 5 cc. of sterile defibrinated rabbit or horse blood.

(4) Mix and pour into Petri dishes or transfer into tubes for slants as desired.

(5) Incubate for 24 hours at 37° to test sterility.

b. *Use of blood agar*

(1) Material to be cultured on blood agar for the isolation or preliminary identification of Pneumococcus may be streaked on the surface of blood-agar plates, either by platinum loops or wires, or by placing a drop of the material on the surface of the medium and then spreading the inoculum over the surface with a bent, sterile capillary pipette. Dilutions of the material in peptone water or broth may be made prior to streaking, depending upon the source.

(2) For colony counts and for studying the activities of pneumococcal colonies in blood agar, the usual procedure is to dilute the culture to an extent that will provide well-isolated colonies, using peptone water as a diluent; measured amounts of the diluted culture, usually 0.5 cc., are transferred to duplicate Petri dishes, and as soon as possible, liquid blood agar at a temperature of 45° to 50° is poured into the dishes. The contents are mixed by gentle rotary motions of the plates, care being taken to avoid splashing or bubbling of the medium.

BLOOD CULTURES (adapted from Zinsser and Bayne-Jones[1579])

Blood is drawn in the usual manner, observing surgical precautions and strict aseptic technique. After the blood is drawn, it should be immediately transferred to the proper medium, for which the usual infusion agar (as referred to under blood agar) and Pneumococcus broth are equally satisfactory.

At least six tubes of agar should be melted and immersed in water at about 45°. Before blood is mixed with the medium, the agar should be cooled to about 41°. The blood is added to the tubes in varying quantities, ranging from 0.25 to 1.0 cc. each, in order that different degrees of concentration may be obtained. Mixing is accomplished by the usual dipping and rotary motion, the formation of air-bubbles being thus avoided. The mouth of each test tube should be flamed before pouring the contents into the plates.

Three flasks of broth suitable for development of Pneumococcus, each containing 100 to 150 cc. of medium, should be inoculated with varying quantities of blood—at least one of the flasks containing the blood in high dilution. It may be advisable to place in the flasks of broth, in addition to glucose, 1 gram of powdered calcium carbonate to inhibit the action of acid on the pneumococci.

For quantitative work, accurately measured amounts of blood should be seeded in blood agar, preferably placing the blood in the Petri dishes, pouring in the melted and cooled agar, and mixing with a gentle rotary motion.

III. Type Determination

MOUSE METHOD (Sabin[1208])

One cc. of a fresh sample of sputum is injected intraperitoneally into a mouse. From 3 to 4 hours later, some of the peritoneal fluid is obtained by puncture with a glass capillary. A glass slide is marked off

into four parts, and a minute drop of the peritoneal fluid is expelled on each one of the four partitions. The first is smeared with saline solution for control, and the others with a loopful of 1:10 dilution of Type I and of Type II, and a 1:5 dilution of Type III diagnostic serums, respectively. These dilutions of serum are chosen largely to eliminate group agglutinins. Thin smears are made, allowed to dry, and fixed by passing the slide through a flame; they are then stained for from 20 to 30 seconds with a fuchsin solution (10 cc. saturated alcoholic solution of basic fuchsin plus 90 cc. of water) or any other available stain. The stain is washed off in water or 20 per cent copper sulfate solution, and the smears are examined with the oil immersion lens. If a specific agglutination reaction is observed in one of the smears with diagnostic serum, the organism is of that type. If no reaction occurs in any of the smears and numerous pneumococci are clearly seen, a diagnosis of Group IV is suggested. When it is desired to know whether the organism is one of the fixed types of Group IV (especially those for which concentrated antiserums are available) a similar procedure is carried out with the homologous immune serums.

Bacteria other than pneumococci in the sputum as well as avirulent forms of pneumococci may occur in clumps in the peritoneal exudate; but these differ in appearance from those produced by specific agglutination and can be distinguished further by their occurrence in the saline control smear. Unless a fresh sample of sputum is used, many of the organisms will have undergone autolysis, and therefore more time must be allowed for growth. Since the mouse is not killed, another type determination can be made if the first should show insufficient organisms, and then after the death of the mouse, the type may be confirmed.

In the case of Type III pneumococci, sufficient organisms are usually present even two hours after injection. The appearance of the specific reaction with Type III pneumococci, primarily on account of the larger size of the capsule, differs somewhat from that obtained with pneumococci of other types; the organisms are farther apart in the agglutinated clumps which occur in mucoid strands. When diagnostic serums for pneumococci of types formerly in Group IV are used, a dilution must be chosen that fails to show any cross-agglutination with any other type.

The peritoneal exudate may contain too many organisms four hours after inoculation or after death of the mouse, so that it may be necessary to dilute the fluid in the capillary tube with saline solution in order to obtain a correct typing. This method is illustrated in Chart 1.

KRUMWIEDE METHOD (Krumwiede and Valentine[762])

A small amount of sputum (3 to 10 cc.) is transferred to a test tube and placed in boiling water for a few minutes until coagulation occurs. The coagulum is broken up with a heavy platinum wire or glass rod and sufficient saline solution added to give just enough fluid after centrifuging. The suspension is again placed in boiling water for a few minutes to extract the soluble antigen from the coagulum, and is shaken several times during the heating process. The mixture is centrifuged and the clear supernatant fluid used for the test.

For the test, 0.2 cc. amounts of undiluted typing serum are placed in narrow test tubes and the antigen carefully layered over the serum. The tubes are placed in a water-bath at 50° to 55° and examined after several minutes. A definite contact ring occurs in the tube containing homologous serum if the sputum is rich in antigen. The true ring is more or less opaque and in the majority of positive reactions is evident in less than 10 minutes. The success of the test depends upon the quality of the sputum. If coagulation does not occur, there is little use in continuing the test (see Chart 1).

NEUFELD QUELLUNG METHOD (Beckler and MacLeod[96])

There are variations in the application of the Neufeld *Quellung* phenomenon to the determination of pneumococcal types in sputum samples. The one given here is the technique used in the Bacteriological Laboratory of the Massachusetts Department of Public Health.

Upon receipt of the sputum at the laboratory, stained liquid mounts of the specimen are mixed with undiluted rabbit antiserums (Types I to XXXII). Combinations of monovalent antiserums (rabbit) are used instead of making separate preparations of the sputum with each of the thirty-two monovalent serums. The combinations of serums used are:

> Type I
> A, Types II, IV, V, and VII
> B, Types III and VIII
> C, Types IX, XI, XIII, and XV
> D, Types VIa, VIb, XVII, and XVIII
> E, Types XII, XIV, XVI, and XXVIII
> F, Types X, XIX, XX, and XXI
> G, Types XXII, XXIII, XXIV, and XXV
> H, Types XXVII, XXIX, XXX, XXXI, and XXXII

Nine loopfuls of sputum are placed approximately one inch apart on a 9 by 2 inch glass slide; to each drop are added 2 loopfuls of the anti-

Krumwiede and Mouse Typing

Sputum

Krumwiede

Emulsify with Saline in morter

Water bath—heat to coag-ulate and extract soluble substance.

Inject into live mouse

Sputum extract layered over typing serum.

I II III

After 3-5 hrs. abdomen punctured—exudate ob-tained.

A cloud at juncture of two fluids is a positive reaction. (type I in this case). Failure of reaction to develop is of no significance as to type of organism.

Sabin

Small drops of exudate smeared on slide with saline and typing sera.

After 5-24 hrs.—averaging 6-8 hrs.—mouse dies or is killed and autopsied.

Wash peritoneal cavity with 5 c.c. saline.

Saline I II III

Stain and examine with microscope.

Centrifuge washings and pipette off supernatant for precipitin test.

Precipitin

Positive reaction showing agglutination in serum of homologous type. Failure of agglutination to develop is suggestive of group IV—but not definite.

Suspend sediment in saline-place in tubes containing equal amounts of typing sera.

Layer supernatant over typing sera.

I II III Sil.

I II III

Shake and incubate 1 hr.

Positive reaction at juncture of the two fluids indicates type. (III in this case). Failure of reaction to occur means group IV.

I II III Sil.

Positive reaction (agglutination) in tube indicates type of organism. (II in this case). Failure of agglutination to occur means pneumococcus group IV.

Krumwiede Typing	Sabin Typing	Tube agglutination Typing	Precipitin Typing
Time 15-20 min.	Time 3-5 hrs.	Time 5-24 hrs. averaging 6-8 hrs.	Time—same as tube agglutination.

JHM

Courtesy of Massachusetts Department of Public Health

CHART I. KRUMWIEDE AND SABIN METHODS OF TYPE

DETERMINATION

Neufeld Typing

Sputum

Transfer fleck of sputum to slide—
mix with typing sera (rabbit)
and alkaline methylene blue.

I   II   III
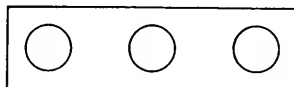
Cover drops with cover
slips and examine under
oil immersion lens with
light partially dimmed.
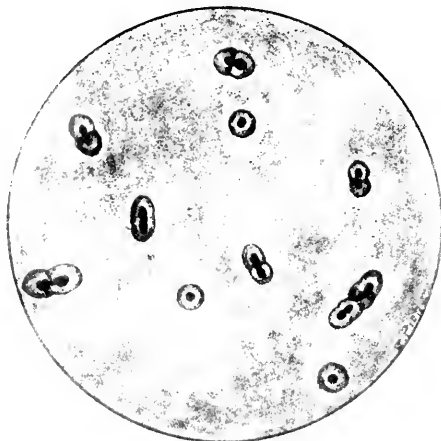
Positive Reaction showing
swollen capsules in prepara-
tion with serum of homol-
ogous type.
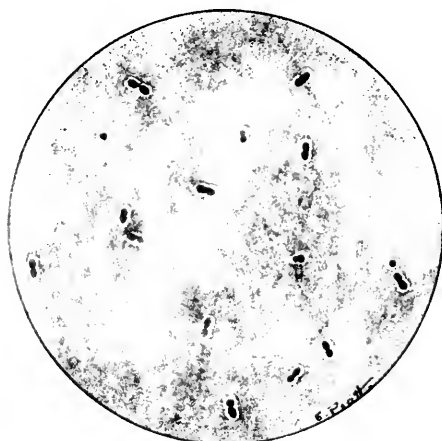
Negative Reaction (capsules not
swollen)
The absence of swollen capsules
in all 3 preparations suggests
Group IV pneumococci.

Time 5–30 Minutes

CHART 2. THE NEUFELD OR QUELLUNG METHOD OF TYPE DETERMINATION

serum, that is, the first drop is mixed with Type I antiserum, the sec-
ond drop with combined serum A (II, IV, V, and VII), the third drop
with B, et cetera. The preparations are stained with Loeffler's alkaline
methylene blue (2 drops to each mixture) and are covered at once with
cover-slips to prevent drying. Examination is made with the oil immer-
sion lens, with the light dimmed. When a positive reaction occurs, which
is usually within a few minutes, there is a decided swelling of the cap-
sule of the pneumococcus present. The swollen capsule is of a light
greenish-gray color, is much less translucent than one that is not swol-
len, and has a definite outline which is one of the most characteristic
features of a positive reaction. In the preparations in which no reaction
is evident, the capsule of the pneumococcus appears as a halo of re-
fracted light. In all preparations the body of the pneumococcus stains a
definite blue. If the reaction is observed in drop 1, then the pneumococ-
cus present is Type I and can be reported immediately; if the reaction
is observed in drop 3, for example, the test is repeated using 2 drops of
the sputum mixed with Type III and Type VIII undiluted monovalent
rabbit serums, respectively; if the reaction is observed in drop 9, five
loopfuls of the sputum are mixed with Type XXVII, XXIX, XXX,
XXXI, and XXXII undiluted monovalent serums, respectively. Should
no reaction be seen on the first examination, the preparations are re-
examined at the end of 30 minutes.

When dealing with sputums containing many Type III organisms, it
is necessary occasionally to dilute the sputum with salt solution before
any *Quellung* of the pneumococcus capsule becomes evident. In such in-
stances, when the regular Neufeld technique is used, the organisms form
large masses surrounded by much precipitated material, and no definite
swelling of the capsules can be seen. However, upon dilution of the
sputum and repetition of the test, the individual diplococci will usually
show a typical, positive reaction.

The age of the specimen of sputum makes little difference in relation
to the success with which the Neufeld method is applied. Positive reac-
tions can be demonstrated on sputum 48 hours or more after collection
from the patient. Chart 2 shows the steps in the technique.

URINE TEST (Dochez and Avery[321])

a. *Unconcentrated urine.* A specimen of urine is obtained as soon as
possible and cleared by centrifuging. The clear urine is mixed in quan-
tities of 0.5 cc. each with equal amounts of antipneumococcic serum of
the different types in a series of agglutination tubes.

The intensity of the reaction may vary from an almost imperceptible cloud to a heavy, flocculent precipitate. The reaction may occur in some instances immediately on mixing the urine and serum, or it may require incubation in the water-bath at 37° for one hour. Prolonged incubation, however, must be avoided, since bacterial growth may obscure the test. In the precipitin reaction it is essential that all the reagents used, including the immune serums, should be water-clear. In case the reaction is negative or so faint as to be indefinite with the whole urine, the following method of concentrating the urine may be employed:

b. *Concentrated urine for precipitin test.* Twenty-five cc. or more of a 24-hour specimen of urine, with the addition of a few drops of acetic acid, are evaporated to a volume of about 5 cc., filtered through paper to remove any precipitate of albumin that may occur, and the filtrate is added to 8 to 10 volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifuging and rapidly dried to remove the excess of alcohol, and the residue extracted with 2 to 3 cc. of salt solution, which redissolves the specific substance. Any undissolved material is removed by centrifuging, and the clear salt solution extract is used for the precipitin test. Chart 3 shows the procedure.

IV. *Isolation of Several Components of Pneumococcus*

MEDIUM FOR THE PRODUCTION OF CULTURE OF STANDARD MAXIMAL DENSITY, VIRULENCE, AND POLYSACCHARIDE CONTENT (O'Meara and Brown[1031])

Witte peptone	10.0 grams
Difco proteose peptone, Fairchild, Parke-Davis, or Neopeptone	40.0 grams
Glucose	5.0 grams
Sodium chloride	5.0 grams
Sodium bicarbonate	5.0 grams
Potassium phosphate (K_2HPO_4)	2.0 grams
Thioglycollic acid	0.1 grams
Distilled water	to 1000.0 cc.

The Witte peptone and sodium chloride are dissolved in water and the reaction of the solution adjusted to pH 8.0. The sodium bicarbonate, glucose, potassium phosphate, and extra peptone (usually Difco proteose) are then added and when they have dissolved the broth is clarified by passing through filter paper. Thioglycollic acid is finally added and the medium sterilized by candling. During the entire course of preparation the temperature of the medium is not allowed to rise above 50°.

Urine Typing

Urine specimen

Centrifuge to clear of debris

Layer clear urine over typing sera

I II III

Positive reaction in form of a pre-
cipitate — generally within a few
minutes—in tube of homologous type.
(type II in this case).
Failure of reaction to occur is of no
significance as to type of organism.
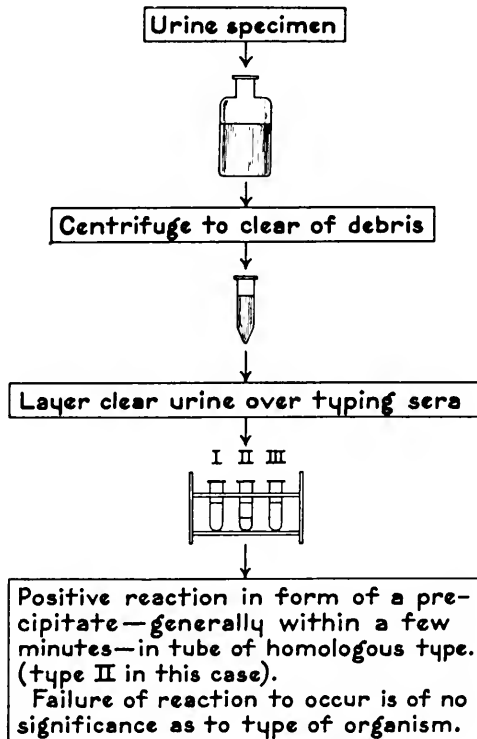
Urine precipitin typing
Time–15–20 minutes.

CHART 3. THE URINE METHOD OF TYPE DETERMINATION

If the medium is allowed to stand for a time, the thioglycollic acid becomes oxidized, and therefore a sterile solution of the acid should be available and an amount sufficient to give a concentration of 0.01 per cent should be added immediately before use. To obtain a culture of standard density a small inoculum from blood broth is transferred to a tube of the medium and incubated overnight. This is then subcultured into fresh medium at 37° and incubated for 5 to 6 hours.

PNEUMOCOCCAL PROTEIN (Avery and Morgan[54])

Concentrated suspensions of organisms in 0.002 N NaOH are subjected to repeated freezing and thawing to disrupt the cell bodies. This thick solution of dissolved organisms is then diluted with salt solution to one-tenth the volume of original culture fluid and passed through a Berkefeld V filter. To this filtered solution N acetic acid is added slowly and the mixture carefully shaken. Flocculation of the protein occurs promptly and completely at a reaction faintly acid to litmus. The precipitated protein is separated by centrifugation, washed several times in distilled water, and redissolved in salt solution by adding 0.1 N NaOH until the solution is faintly alkaline to litmus. Solutions of pneumococcal protein freshly prepared by this method exhibit the usual qualitative color reactions for substances of this nature: positive biuret, Hopkins-Cole, Millon, xanthoproteic, and Molisch reactions. The hydrolyzed protein gives the purine reaction with Fehling's solution. The protein solutions are standardized on the basis of their nitrogen content.

SOMATIC CARBOHYDRATE OR C FRACTION (Tillett, Goebel, and Avery[1410])

The bacterial cells from 3 liters of broth culture of an R strain derived from Type II Pneumococcus were collected by centrifugation. The bacteria were resuspended in 50 cc. of salt solution and were repeatedly frozen and thawed to break up the bacterial bodies. To this solution of bacteria 0.5 cc. N acetic acid was added, and the mixture was then heated for 10 minutes in a boiling water-bath. The tube was cooled and the coagulated protein was separated from the clear supernatant liquid which contained the C substance. In this manner the non-coagulable material from 30 to 40 liters of culture was collected. The combined supernatant extract, after neutralization, was finally filtered through a Berkefeld candle and then concentrated *in vacuo* to 50 cc. The concentrates from 36 liters of bacteria obtained as described above

were again acidified with 0.5 cc. of N acetic acid and reheated for 15 minutes at 100°. A small amount of coagulated protein was thus separated and removed by centrifugation. The clear solution was now treated with 5 volumes of alcohol and after standing overnight in the ice-box, a precipitate settled out which contained all of the so-called C substance, together with certain nitrogenous impurities. This precipitate, when redissolved in saline solution, was found to give a specific precipitin test with antipneumococcic horse serums of Types I, II, and III. The solution of the C fraction was found to contain, however, material which gave a positive biuret test, but this impurity was precipitated from solution by making the mixture alkaline with sodium hydroxide, without loss of the serologically reactive substance. The alkaline solution of the C fraction, now at a volume of 50 cc., was reprecipitated by the addition of 5 volumes of alcohol. The material was centrifuged, redissolved in 40 cc. of water, and again precipitated from faintly acid solution by alcohol. This procedure was repeated altogether four times. The carbohydrate recovered from the final alcoholic precipitation was dissolved in 15 cc. of water and was cooled to 0°. To the solution were added 2 cc. of hydrochloric acid (sp. gr. 1.09). A small amount of insoluble inactive material separated from the solution and was centrifuged off. The clear acid solution was now precipitated with five volumes of redistilled alcohol. After standing at 0° for 4 hours the C substance was separated by centrifugation. It was redissolved in 10 cc. of water and then reprecipitated with alcohol and acid. The final product was washed free from chlorides with 85 per cent alcohol, and was washed finally with redistilled alcohol and ether. The yield was about 65 milligrams from 36 liters of broth cultures.

CAPSULAR POLYSACCHARIDE (Heidelberger, Kendall, and Scherp[627])

a. *Preparation of the specific polysaccharide of Type I Pneumococcus.* 10 liters of phosphate meat-infusion broth containing 0.3 per cent of added glucose were seeded with a highly virulent Type I pneumococcus.* After 72 hours at 37°, 1 per cent of phenol was added and the culture allowed to stand overnight. It was then centrifuged in a Sharples centrifuge and the effluent concentrated to 1 liter under reduced pressure, keeping the temperature below 35°. 100 grams of crystalline sodium acetate were dissolved in the concentrated broth and 1,250 cc.

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* Recent mouse passage is necessary for good yields of the polysaccharide. After a period of 3 weeks between the last mouse passage and the preparation of the polysaccharide the yield was reduced 80 per cent.

of 95 per cent alcohol were added with constant stirring. The polysaccharide separated as a white curdy mass which settled rapidly. After standing overnight the supernatant fluid was poured off and the precipitate centrifuged. Usually a three-layer separation was obtained, but in cases in which only a single volume of alcohol was used, the phosphates and other salts were not thrown out and no syrupy layer was found. The three-layer separation was also not obtained in the absence of phosphates. If the broth used is phosphate-free, purification of the polysaccharides may often be greatly facilitated by addition of sufficient phosphate to bring about the three-layer separation. A solution of 10 grams of sodium acetate in 250 cc. of water was made acid to litmus with acetic acid and the alcohol precipitate (or middle layer) was dissolved in this and reprecipitated with 300 cc. of 95 per cent alcohol. The centrifuged precipitate was dissolved in 250 cc. of water containing 10 grams of sodium acetate and 5 cc. of glacial acetic acid, and the turbid solution was shaken with 50 cc. of chloroform and 10 cc. of n-butyl alcohol for 30 minutes. On centrifugation a semi-solid emulsion separated between the aqueous layer and the chloroform. The solution and the chloroform were poured off and the layer of emulsion was washed with two 50 cc. portions of water which were saved for washing later emulsion layers. 50 cc. of chloroform and 10 cc. of butyl alcohol were added to the aqueous layer and the shaking was repeated. On centrifugation a smaller semi-solid emulsion layer was formed and this was treated as before. Shaking with chloroform was repeated as long as an emulsion layer formed. Seven shakings were usually required. The washings of the emulsion layers were combined and shaken with fresh additions of chloroform as long as an emulsion layer formed, and were then combined with the main aqueous solution, the total volume now being 350 cc. The polysaccharide was precipitated with 500 cc. of 95 per cent alcohol, redissolved in 250 cc. of water, and the solution tested for phosphate and glycogen. Phosphate may be removed either by repeated precipitations with alcohol in the presence of sodium acetate and acetic acid or with glacial acetic acid in the presence of sodium acetate. Glycogen may be left behind by precipitating the SI from a salt-free aqueous solution with copper acetate. In the instance quoted the solution was free from phosphate but gave a strong iodine test for glycogen. 20 cc. of a saturated solution of copper acetate slightly acidified with acetic acid were added and the resulting bluish precipitate was centrifuged off. The supernatant fluid remained clear when more copper acetate was added. The precipitate was dissolved in 50 cc. of 20 per cent sodium

acetate solution and 5 cc. of glacial acetic acid, and reprecipitated free from copper, after which it was dissolved in 100 cc. of water and the solution again tested for glycogen. If this is present the copper precipitation is repeated. The SI was finally precipitated with redistilled alcohol in the presence of a small amount of sodium acetate, washed with redistilled alcohol, filtered, and dried. Yield: 0.9 gram of the neutral sodium salt of SI.

b. *Preparation of the specific polysaccharide of Type II Pneumococcus.* 10 liters of a 4-day culture of Type II Pneumococcus were treated in the same way as for Type I. No differences were observed up to the point at which the copper acetate precipitation was made. However, only one-third of the SII was thrown down as the copper salt. This fraction, which was free from glycogen, was reprecipitated as the copper salt and isolated separately.

The copper-soluble fraction was freed from copper salts by several precipitations with alcohol in the presence of acetic acid and sodium acetate and was redissolved in a small volume of water. Even this concentrated solution failed to react with copper acetate. As glycogen was present in this fraction it was removed by adjusting the pH to 6.5 and adding a small amount of saliva. After a few minutes the iodine test was negative. To remove protein impurities added in the saliva, 5 grams of sodium acetate and 2.5 cc. of acetic acid were added, and the solution (volume 100 cc.) was repeatedly shaken with chloroform and a little butyl alcohol until an emulsion layer was no longer formed. Since the polysaccharide solution still contained nitrogen it was adjusted to contain 5 grams of sodium acetate per 100 cc. and was chilled and precipitated with 5 volumes of glacial acetic acid. The precipitate was centrifuged in the cold, taken up in 50 cc. of 5 per cent sodium acetate solution, and again precipitated with 5 volumes of acetic acid. This was followed by two precipitations with alcohol from 5 per cent sodium acetate solution and one precipitation with redistilled alcohol, after which the polysaccharide was washed with redistilled alcohol, filtered, and dried. One cc. of the broth contained 0.59 mg. or a total of 0.590 gm. of SII in the 10 liters used. Recovery, 78 per cent.

c. *Preparation of the specific polysaccharide of Type III Pneumococcus.* The culture filtrate was concentrated in vacuo to one-tenth its original volume. After three precipitations with 1.5 volumes of alcohol, part of the protein contained in the material was removed by 40 per cent saturation with sodium sulfate at 37°. The SIII was then precipitated by completely saturating the solution with sodium sulfate. After

removing the sodium sulfate by repeated precipitations of the SIII with alcohol and acetic acid in the presence of sodium acetate the product was found to contain 2.4 per cent of nitrogen. This was removed by twice precipitating the SIII as the barium salt with barium chloride. The barium was removed by repeated precipitations from 20 per cent sodium acetate solution with acetic acid and alcohol. The SIII was isolated as the neutral sodium salt.

V. Preparation of Bacterial Enzymes Capable of Decomposing Capsular Polysaccharides (Dubos,[336] and Dubos and Bauer[338])

The bacteria are grown in a solution of 2 per cent casein hydrolysate (pH 7.0) at 37° under conditions of strict aerobiosis; the cells from the 16-hour-old culture, separated by centrifugation, are resuspended in small amounts of distilled water.

A medium is prepared consisting of 0.1 per cent capsular polysaccharide and 0.1 per cent NaCl in distilled water. This medium is distributed in 25 cc. amounts in large Erlenmeyer flasks (1 liter capacity) to provide for aerobic conditions, and each flask is inoculated with the cells recovered from 500 cc. of the culture of the SIII bacillus in the casein hydrolysate medium. The material is incubated for 12 to 18 hours at 37° and the cultures tested to ascertain the disappearance of the specific polysaccharide and the absence of contaminants. The cultures are now frozen and thawed repeatedly to secure the release of the endocellular enzyme.

The enzyme is ultimately separated from the cell débris by filtration. However, since the cell suspension is very viscous, it is first subjected to the following treatment. The cell suspension is made alkaline to pH 10.0 by the addition of sodium borate. Equimolecular concentrations of dibasic sodium phosphate and calcium chloride are then added to bring about a heavy precipitate of calcium phosphate which facilitates the clarification of the material by centrifugation. The supernatant fluid which contains all the enzyme in solution is now passed through a Seitz filter, then through a Berkefeld (V) filter. The potency of this filtrate is such that 0.002 to 0.004 cc. are required to decompose 0.01 mg. of the capsular polysaccharide under the conditions of the test.

For the purpose of further purification of the enzyme preparation, the solution is passed through collodion membranes made and calibrated according to the description given by Bauer and Hughes.[90] Membranes

with a pore diameter of 5.5 to 7.5 mμ are used in a specially designed filter of stainless steel. Filtration is carried out at a pressure of 50 pounds per square inch. To minimize contamination by air bacteria and particles of dust, the air is filtered through Seitz asbestos pads inserted between the air line and each individual filter. Chloroform is used as an antiseptic during filtration. At the end of filtration (it may take 5 days to filter to dryness 600 cc. of enzyme solution), the membranes are washed several times in distilled water and all the enzymatic activity is thus recovered in solution. After filtration through Berkefeld (V) candles, the preparation is frozen and in this state evaporated to dryness in a vacuum. The final product is used dissolved in water.

VI. Serological Reactions

AGGLUTINATION

Since different lots of serum may exhibit different agglutinin titers, it is advisable to test each batch of serum in varying dilution against a culture of known type-specificity. It may occasionally be necessary, also, to employ dilutions of the material (suspensions of living or killed pneumococci) used as antigen, since zonal effects may inhibit complete agglutination. As a rule, an 18-hour broth culture, containing approximately one billion organisms per cubic centimeter serves as a satisfactory suspension for routine tests.

For the test, it is convenient to mix 0.5 cc. of serum dilutions with an equal amount of the culture. The serum dilutions may range from undiluted, for certain types, to 1 to 5 through 1 to 80 or 160. With unknown types, it may be well to perform a bile-solubility test at the same time. The mixtures are incubated in a water-bath at 37° to 40° and examined at frequent intervals up to two hours. Those tubes showing no agglutination, or an indefinite reaction, should be placed at 4° overnight, and if the reaction is still negative, the tubes are then discarded.

With cultures of strains known to be closely related, it is well to perform tests for heterologous reactions. Thus, a Type V culture should be tested with both Type V and Type II serum, and likewise a Type II serum should be tested against both Type II and Type V cultures.

Antipneumococcic rabbit serums exhibit a higher degree of type-specificity than do horse serums, and for this reason are of value in case of doubt regarding the type-specificity of recently obtained strains.

PRECIPITATION

There are various methods of titrating antipneumococcic serum based on precipitation of the antibody by the specific polysaccharide. Those listed below are given to illustrate the methods in more common use.

a. *Optimal proportions* (Smith[1298]). The test is carried out in three routine stages.

(1) Preliminary Orientation: The object is to decide upon a suitable dilution in which to use the serum in the subsequent tests, for if the serum is used insufficiently diluted, precipitation is so rapid that it becomes impossible to decide which tube is the optimal one. A suitable serum dilution is that which produces macroscopic precipitation with the equivalent amount of antigen in 20 to 30 minutes. Dilutions of serum, 1 in 5, 1 in 10, 1 in 20, are made with 0.85 per cent salt solution and 1 cc. of each mixed with 1 cc. of polysaccharide in 1 in 50,000 dilution. The tubes are placed in the water-bath at 40° and are examined at the end of 5 minutes and again at 10 minutes. The choice of the serum dilution to be used in the subsequent tests, based on the result obtained, is a matter of personal judgment, readily acquired from a few experiments.

(2) Rough Test with Widely Spaced Antigen Dilutions: A row of eight tubes is numbered from left to right and 1 cc. of buffer solution (borate buffer solution, pH 8.0) is run into each. To the first tube, 1 cc. of a stock solution of SSS, 1 in 10,000 dilution, is added by means of an accurate 1 cc. pipette, the contents are mixed thoroughly and 1 cc. of the mixture is carried over to tube 2, and so on, 1 cc. being discarded from the last tube. It is not necessary to use separate pipettes. To each tube in turn, 1 cc. of the chosen dilution of serum is added by means of a mounted syringe, care being taken to prevent the stream of serum from striking the walls of the tube. (A 2 cc. all-glass syringe is mounted vertically on a rigid stand with an adjustable screw over it, so that when the piston is raised to meet the screw, the syringe capacity is exactly 1 cc. A 23-gauge half-inch needle is fitted to the syringe, which can now be filled rapidly and accurately with serum, held in a conical centrifuge tube.) The rack of tubes is placed in the water-bath and transferred periodically for a rapid examination to a reading box, so designed that the whole series of mixtures, evenly transilluminated, can be viewed against a dead black background.

In this rough test, one tube usually shows precipitation long before

any of the others; occasionally two tubes run parallel, indicating that the true optimal antigen concentration lies midway between them.

(3) Final Test with Narrow Range of Antigen Dilutions: Eight tubes are numbered and buffer solution is added from a graduated pipette; nil to tube 1, 0.1 cc. to tube 2, 0.2 cc. to tube 3, and so on. With a fresh pipette, enough antigen solution is added to each tube to bring the total content up to 1 cc. The dilution of SSS solution to be used is decided from the result of the rough test, the object being to have the optimal tube at about the middle of the series. Serum is added in 1 cc. quantities as before and the tubes are incubated. Closer observation is necessary than in the previous test because, with this narrow range of antigen dilutions, precipitation extends rapidly from the optimal tube to those on either side. A hand lens is used until one of the precipitates can be seen by the naked eye, but even so it is often impossible to be sure which tube first shows precipitation. The optimal tube, however, is distinguished more by the fact that in it the particles remain larger for some considerable time than those in any other tube and also the fluid part of the mixture looks clearer and more transparent than in any of the others. The antigen-antibody ratio is then calculated by dividing the antigen dilution of the optimal mixture by the serum dilution.

(4) Confirmation of the Precipitation Result by Tests for Excess of Antigen: Occasionally two, three, or even four tubes run so nearly parallel that it is impossible to be sure which represents the optimal; that is to say, an optimal band is produced instead of an optimal tube. In such cases it is still possible to find the optimal ratio by centrifuging the mixtures after precipitation is complete in all of them, and testing the supernatant fluids for excess of antigen. In practice it is sufficient to show that antigen remains free only in those tubes to the left of the optimal. A simple method is to layer the supernatant fluid over a potent serum suitably diluted in normal horse serum; the formation of a white ring at the junction of the fluids indicates that polysaccharide has remained in the supernatant fluid.

b. *Nitrogen determination* (Heidelberger, Sia, and Kendall[630]). If, on addition of a 1 to 10,000 saline solution of the specific polysaccharide to a portion of the immune serum, precipitation is rapid and heavy, 0.5 cc. of the serum should be used for the quantitative determination. This amount will suffice for all except very low grade serums, of which it is better to use 1 cc. The serums should be measured in duplicate

with accurately calibrated pipettes into wide agglutination tubes (dimensions of 10 by 75 mm. have been found suitable). If 0.5 cc. of serum has been used, 1 cc. of saline solution is then added to each tube, followed by 0.5 cc. of a saline solution containing 1 mg. of the specific polysaccharide per cubic centimeter, to make a total volume of 2 cc. Ordinary uncalibrated pipettes are adequate for the saline and polysaccharide solutions. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the finger tips rapidly and repeatedly in a diagonal stroke down the side of the tube. The tubes are allowed to stand 2 hours in the water-bath at 37° and overnight in the ice-box.* The plugs are then removed and the tubes centrifuged for 10 minutes at 1,000 R.P.M., either in a refrigerating centrifuge or immersed in ice-water. The supernatant liquid is then carefully drained off by inverting the tube and wiping the mouth of the tube with filter paper. The tubes are then placed in ice-water and each precipitate is washed with 2 cc. of an ice-cold, 1 to 20,000 saline solution of the specific polysaccharide, mixing the contents as before. It appears to make little difference whether the disc of precipitate is loosened from the bottom of the tube. After one-half hour in the cold, the tubes are again centrifuged and drained as before. About 0.5 cc. of water is then added to each tube, with shaking until the precipitate is loosened, after which the disc is dissolved by the addition of 2 drops of normal sodium hydroxide solution with rotation of the tube until the precipitate has disappeared. If the disc is allowed to stick to the glass, solution is much slower. The solution is then rinsed quantitatively into a micro-Kjeldahl flask or tube and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, is satisfactory. Nitrogen found multiplied by 6.25 equals specifically precipitated protein.

c. *Modified routine*† (Barnes, Clarke, and Wight[82]). The test is conducted by mixing a constant amount of capsular polysaccharide with varying dilutions of immune serum and comparing the precipitin titer of the unknown serum with that of a control serum tested simultaneously.

(1) Dilution of SSS: Stock solutions of SSS are made up in 1 to

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* Sterile technique should be employed up to this point. In the case of low-grade serums, identical values are obtained if the tubes are allowed to stand in the water-bath only one-half hour, followed by one-half hour in ice-water before proceeding. With more potent serums, results a few tenths of a milligram per cubic centimeter too low are obtained by shortening the process in this way.

† Suitable only for unconcentrated serums.

1,000 dilution with borate buffer solution (pH 8.0). From this stock solution, various dilutions are tested against varying dilutions of a control serum to determine the suitable dilution of the carbohydrate to be used routinely. This dilution will vary from lot to lot and according to the type of SSS. To illustrate, for Type I, the dilution 1 to 30,000; for Type II, 1 to 80,000; for Type V, 1 to 50,000; and for Type VIII, 1 to 80,000 may be found to be satisfactory.

(2) Dilution of Serums: Serums are diluted with physiological salt solution in series, 1 to 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 160, 200, 240, 320, 400, and so on. For the test, ten adjacent dilutions from the series are employed.

(3) The Test: 0.5 cc. of the dilution of SSS chosen is added to 0.5 cc. of the serum dilutions, and the contents mixed by shaking the racks for several minutes. The mixtures are incubated for two hours at 40° and overnight in the refrigerator at 4°.

(4) Interpretation: The end point is taken as the highest dilution of serum showing definite precipitation. By comparing the titer of the unknown serum with that of the control, the number of units in one cubic centimeter of the unknown serum can be calculated.

d. *Combining equivalents* (Felton and Stahl[433]).

(1) Preparation of SSS Dilutions: The stock solution is made in a volumetric flask from an accurately weighed sample of the dried material, dissolving it first in a small volume of saline solution with the addition of sufficient normal NaOH to maintain a neutral reaction and facilitate solution. Then salt solution is added to complete the volume for a 1 to 500 dilution. Using a volumetric flask and a quantitative delivery pipette, the dilution required for the test is prepared in such a quantity that not less than 1 cc. of the 1 to 500 stock solution need be measured.

(2) Preparation of Serum Dilutions: Physiological salt solution for the serum dilutions is measured with a burette when more than 2 cc. is required, and with a 2 cc. accurately calibrated serological pipette, graduated in tenths, when less than 2 cc. is needed. Serum for the initial dilution is drawn up to the line in a quantitative pipette calibrated to contain exactly the amount required. Excess serum clinging to the outside of the pipette is removed with a piece of cotton or gauze, and the pipette is rinsed five times in the dilution being made. Two cc. serological pipettes graduated in tenths are used in making subsequent dilutions. Each dilution should be in at least 1 cc., and preferably 2 cc. or more, volumes.

(3) Mixing the Two Components (first step): After the serum dilutions have been properly prepared, quantitative pipettes are used to deliver 1 cc. volumes into small agglutination tubes (10 cm. by 1 cm.) arranged in suitable racks. Then, with a quantitative delivery pipette, 1 cc. of the chosen SSS dilution is added to each, and the tubes are shaken separately to ensure thorough mixing. The tubes are incubated for 2 hours in a 37° water-bath, in which the water extends half-way up to the level of the mixtures in the tubes, and then stored in the cold (4°) overnight.

(4) Adding the Indicating Serum (second step): The next morning, the precipitates are separated from the supernatant fluids by. centrifugation for 3 or 4 minutes, preferably in the cold or at room temperature. The supernatant fluids are drained into clean, clear agglutination tubes and to them is added the indicating serum in the amount previously found to be optimal, and each tube is shaken thoroughly. The mixtures are incubated at 37° for 2 hours, and then, without disturbing the precipitates, allowed to settle for 2 hours at room temperature before recording the end point.

(5) Reading the End Point: For consistent and reliable results, the end point is considered as that dilution next in series to the one containing a well-formed disc precipitate. With some serums and some SSS preparations, the end point is extremely definite, that is, of two adjacent tubes in a series, one contains a disc precipitate, the other contains not even a trace of flocculation. On the other hand, there are instances when the mixtures next in series to the last disc precipitate are not clear, but contain a fine sediment which becomes a swirl when the tube is shaken. These fine precipitates are disregarded, and the tube following the last disc precipitate in the series is taken as the equivalent end point.

(6) Calculating the Unit Value: The greater the unit value of any antibody preparation, the smaller is the volume required to combine equivalently with a given dilution of specific carbohydrate. Hence, an inverse proportion expresses the relationship between the antibody content of an immune serum and the percentage dilution that represents its equivalent end point. The unit value of an unknown serum is found from the proportion

$$A : A' = B' : B, \text{ or } A = \frac{A'B'}{B}$$

in which A equals the unit value of the unknown serum, A′ the unit

value of the standard serum, B the equivalent end point of the un-
known, and B′ the equivalent end point of the standard serum.

COMPLEMENT FIXATION (Goodner and Horsfall[538])

As expressed by Goodner and Horsfall, "Complement is not fixed by
immune aggregates resulting from the interaction of pneumococcus cap-
sular polysaccharide and type-specific immune horse serum, although
under proper conditions the substitution of immune rabbit serum gives
positive results." The materials and methods used by these authors are
as follows: Fresh guinea pig serum (complement), a 5 per cent suspen-
sion of washed sheep red blood cells, and the serum of a rabbit im-
munized with sheep red blood cells (amboceptor). Each reagent is used
in a volume of 0.5 cc. The amboceptor is titrated with excess of comple-
ment and so diluted that 0.5 cc. contains 2 units. The complement is
titrated against 2 units of amboceptor. The final volume in each tube of
the titrations is 1.5 cc. The majority of the experiments were carried
out with the acetyl form of Type I pneumococcus capsular polysaccha-
ride.

For further details of the tests the reader is referred to the original
article by Goodner and Horsfall.

BACTERICIDAL TESTS (Ward[1480])

In this method, a constant amount of whole blood is placed in a series
of tubes, and to each of the tubes is added a decreasing number of or-
ganisms, so that—for example—the first tube is inoculated with 500,-
000 organisms, the second tube with 50,000 organisms, the third tube
with 5,000 organisms, and so forth. The tubes are sealed and placed in
a rotating box in the incubator. After some hours' incubation, the tubes
are opened and the contents plated. The plates are incubated and read
the next day. It is evident that the sterility of the contents of the tubes
—as shown by the plates—depends on three main factors, namely, the
number and virulence of the organisms, and the phagocytic power of the
whole blood. As the number of organisms can easily be controlled, the
method can be used to determine either the virulence of the organisms,
by always using the same blood, or the phagocytic power of the blood,
by always using the same organisms, providing their virulence is main-
tained at a constant level. The maximal number of organisms killed in
the mixture is, in the one case, the direct measure of the phagocytic
power of the blood and, in the other case, the indirect measure of the

virulence of the organism. In other words, the more organisms that are killed, the more actively phagocytic is the blood in the former case, and the less virulent is the organism in the latter case.

The method is a modification of the one described by Todd. For further details, the reader should consult the original article by Ward.

VII. Potency Tests on Antipneumococcic Serum

UNITED STATES HYGIENIC LABORATORY, NOW NATIONAL INSTITUTE OF HEALTH (as of January 22, 1926)

a. *Culture.* The culture of Type I pneumococcus should be of such virulence that it is fatal to mice, as a rule, in 0.00,000,001 cc. of a 3 to 6-hour beef infusion broth culture. Simultaneously with each day's tests, eight mice are inoculated with the culture alone, receiving no serum, in order to check the virulence of the culture used. These cultural check mice receive 0.00,000,1 cc., 0.00,000,01 cc., 0.00,000,001 cc., and 0.00,000,000,1 cc. of the culture, two mice on each dose. These dilutions are made as follows:

Flask or tube	Culture	Sterile broth	Each 0.5 cc. of original culture contains
No. 1	1 part	4 parts	0.1 cc.
No. 2	1 cc. of No. 1	9 cc.	0.01 cc.
No. 3	1 cc. of No. 2	9 cc.	0.00,1 cc.
No. 4	1 cc. of No. 3	9 cc.	0.00,01 cc.
No. 5	1 cc. of No. 4	9 cc.	0.00,001 cc.
No. 6	1 cc. of No. 5	9 cc.	0.00,000,1 cc.
No. 7	1 cc. of No. 6	9 cc.	0.00,000,01 cc.
No. 8	1 cc. of No. 7	9 cc.	0.00,000,001 cc.
No. 9	1 cc. of No. 8	9 cc.	0.00,000,000,1 cc.

Either conical flasks or tubes 28 mm. in diameter are used for making these dilutions. Thorough mixing is very essential and this is accomplished only by a rapid and vigorous blowing and sucking of the mixture with a minimal amount of air through the pipette, which is pressed against the bottom of the flask or tube. A separate pipette is used for each dilution. The culture should be checked for purity by plating on blood-agar plates previous to each test. At the Hygienic Laboratory the broth for growing the cultures is made from beef infused in the ice-box, and 2 per cent peptone is used. The hydrogen ion concentration is about pH 7.2. The culture is passed through mice weekly, 0.5 cc. of an 18-hour culture being inoculated intraperitoneally into each of three mice,

which are etherized in 5 or 6 hours, about 0.5 cc. of heart's blood being obtained from each and distributed into two tubes of broth and a blood-agar plate (for purity test) which are incubated overnight. Three of the six tubes showing a good growth of Pneumococcus in pure culture are selected and stored at 15° as stock cultures; after about 3 days (the day before the test) 0.5 cc. to 1.0 cc. are transplanted from each stock culture to fresh tubes of broth, and plates are made for purity. The stock cultures are quickly put back at 15°, to be used for planting the tubes 18 hours before the next passage through mice. The first trans-plants are incubated 16 to 18 hours, transplanted again, and incubated 3 to 6 hours, and of these three last transplants, those showing a good growth of pure Pneumococcus, with a turbidity of 200 to 250 parts per million, silica standard, and a pH of 6.6 to 7.0 are pooled and distrib-uted for the test. All dilutions of cultures are made in broth and should not stand diluted for more than an hour at room temperature.

0.1 cc. of the culture, diluted so that this dose is contained in 0.5 cc. volume (tube 1 in the above table), is given to each mouse, except the cultural check mice.

b. *Serum.* 0.2 cc. of each serum (including the control serum) diluted with saline solution so that this dose is contained in 0.5 cc. (2 volumes serum diluted and mixed thoroughly with 3 volumes saline) is given to each mouse, except the cultural check mice.

c. *The test.* For each serum tested, at least three mice are injected intraperitoneally with 0.2 cc. of serum, followed in less than 5 minutes by 0.1 cc. of culture intraperitoneally. Six mice should receive the con-trol serum, three at the beginning and three at the end of each test. The mice should weigh 18 to 22 grams. If there is much variation in weight, they should be arranged in three weight groups, and a light, a medium, and a heavy mouse chosen for each serum. The mice are observed for 96 hours; all mice counted as fatalities must be proved, by smear and cul-tures from the heart's blood, to have died with an uncomplicated pneu-mococcal septicemia. Survivals of less than twelve hours should be re-garded as "contaminations." Mice dying of infections or accidents other than pure pneumococcal septicemia, as revealed by necropsy, are re-garded as "contaminations." Contaminations should be regarded as neither deaths nor survivals, but as if not injected. Tests are read 120 hours after injection, to allow for the obtaining of results of necropsies on mice dying on the fourth day. The control serum should, on the aver-age, protect at least 50 per cent of the mice. The following data should be included in the protocols of tests:

Date of test, including month, day, and hour
Operator's initials
Date of last animal passage of the culture
Period of exposure of diluted culture to room temperature
Period of time necessary for injection of all mice
Hours of survival of mice dying up to 96 hours, indicated by number
Survival of mice for 96 hours, indicated by S
Contamination to be indicated by circle around the number of hours of survival

SAMPLE PROTOCOL OF MOUSE PROTECTION TESTS, NATIONAL
INSTITUTE OF HEALTH

LEAGUE OF NATIONS (as taken from Parish[1051])

The test is intended to ascertain the amount of serum that is equivalent in protecting power to a certain dose of a standard serum, the serum and culture being mixed for 5 minutes at room temperature and injected intraperitoneally in 1.0 cc. amounts of mixture. The culture dose suggested by the League of Nations' Subcommittee is 0.001 cc. In each experiment, varying dilutions of serum in 0.9 per cent saline solution are mixed with equal volumes of the culture dilution in broth. Decreasing amounts of serum are used, such as 0.001, 0.00,05, and 0.00,025 cc.

a. *Animals.* The mice selected for the tests should weigh 18 to 22 grams. After the injections of serum and culture, the dead and sick animals should be noted morning and evening. Smears are made from the hearts' blood of the dead mice and only those animals showing the presence of large numbers of pneumococci are counted as fatalities for the

purposes of the titration. Mice failing to show pneumococci in smears of hearts' blood are regarded as neither deaths nor survivals, but as if not injected.

b. *Culture*. The culture of Type I Pneumococcus should be of such virulence that it is fatal to mice in doses of 0.00,000,001 to 0.00,000,-000,1 cc. (16 to 18-hour culture in steamed Witte peptone broth at a pH of 7.5). At the beginning of each day's tests, mice are inoculated intraperitoneally with 1 cc. of 0.00,000,000,1, 0.00,000,001, and 0.00,000,1 of the culture, two mice with each dose, to check the virulence of the organisms.

For the maintenance of virulence, cultures should be passaged through the mouse monthly, being kept between passages in 50 per cent defibrinated horse or rabbit blood broth (not incubated) in 1.0 cc. vials in the ice-chest at 4°. When required for test, the cultures are incubated and used as the inoculum for 5 cc. or 10 cc. tubes of Witte peptone broth. A stock of Witte peptone broth which is known to be satisfactory should be kept for the tests, and before it is expended another supply should be prepared and tested in parallel with the stock before being adopted for use.

c. *Serum*. In every test a standard control serum should be available for purposes of comparison. Dilutions of this serum and of all serums to be tested are made in 0.9 per cent saline solution, a different pipette being used for each dilution.

AUTHOR'S NOTE: Parish recommends the use of 0.01 cc. of an 18-hour broth culture rather than 0.001 cc. as the optimal dose for routine use.

AMERICAN DRUG MANUFACTURERS' ASSOCIATION

The method was suggested by the Committee on Standardization of Antipneumococcic Serum of the Biological Section of the American Drug Manufacturers' Association, February 21, 1933, and revised December 29, 1933.

a. *Unit of serum*. The unit of antipneumococcic serum shall be 1/200 cc. of F–146 or its equivalent which is 1/300 cc. for Type I and 1/150 cc. for Type II of P–11. In the performance of the test, the dose of serum used is the unitage tested for. In using the control serum P–11 at a unitage of 300 units per cubic centimeter for Type I and 150 units per cubic centimeter for Type II, which is the accepted potency of this control serum, 0.5 cc. of a 1:150 dilution for Type I and of a 1:75 dilution for Type II is injected.

b. *Test dose of culture*. The test dose of culture is that amount of culture which, when drawn up in the same syringe with a unit of con-

trol serum and injected into the test mice, will on the average kill approximately 40 per cent of the mice injected. This test dose shall be not more than 0.5 cc. of a 1:100 dilution. With larger amounts of serum a higher percentage of mice is expected to live, and with smaller amounts of serum, a lower percentage of mice is expected to live. The culture to be used is one found by experience to be satisfactory for use for test with the control serum.

c. *The test.* At least two complete tests, preferably made on different days, using 10 mice on each serum dilution, must be made for each unknown serum.

(1) Mice: 16 to 20 gram white mice are used for the test. Ten mice are injected on each serum dilution.

(2) Serum: The serum is diluted, at room temperature, with physiological salt solution, never using less than 1 cc. volume in making dilutions, except if economy requires, in the first dilution of the control serum, when 0.5 cc. may be used. Accurate quantitative pipettes, with a separate pipette for each dilution, are employed.

(3) Control serum: (see Unit of serum, *ante*). It is essential that the control serum P–11 be used in at least 2 dilutions, 300 and 400 units for Type I and 150 and 200 units for Type II, namely 0.5 cc. of 1:150 and 0.5 cc. of 1:200 for Type I, and 0.5 cc. of 1:75 and 0.5 cc. of 1:100 for Type II, with each comparative test. On the average, in at least two tests, 60 per cent of the mice must survive in the 300 unit dilution and over 50 per cent die in the 400 unit dilution in Type I, and 60 per cent of the mice survive in the 150 unit dilution and over 50 per cent die in the 200 unit dilution in Type II.

The dilution of the unknown serum depends upon the relative potency. Suggestion: A primary titration may be made using mice in triplicate on dilutions representing 100, 200, 400, 800 units, etc., when testing unconcentrated serum, or 1,000, 2,000, 3,000, 4,000 units, etc., in refined and concentrated serums or globulins. After establishment of the approximate unit value of the serum, dilutions in the appropriate zone with an increment of 25 to 50 per cent are tested with ten mice to each dilution.

(4) Culture: (see Test dose of culture, *ante*).

(5) Method of Injection: In actual test, 0.5 cc. of serum dilution is drawn into a 1 cc. tuberculin syringe (fitted with a No. 23 gauge, ¾ inch needle) and then 0.5 cc. of the culture dilution is drawn into the same syringe and the mixture injected immediately (within 5 seconds). A different syringe must be used for each dilution of serum. For each dilution of serum tested a separate container shall be used

for the culture dilution. Not more than 60 minutes shall elapse be-
tween starting and finishing the injections.

(6) Length of Test: The test is terminated at the end of 96 hours
and all mice living at the end of this time are counted as survivals.
The hour of death is noted on mice dying before 96 hours. (Ideally,
all mice dying on the test should be necropsied and the hearts' blood
cultured to rule out deaths due to intercurrent infection. This may be
impractical in routine testing, but certainly in each test some mice on
important dilutions should be examined post mortem.)

d. *Interpretation of test.* The unit value of an unknown serum is esti-
mated by direct comparison of the highest or end-point dilution pro-
tecting 60 per cent of mice on an average in at least two complete and
separate tests with the control serum P-11 end-point dilution equalling
300 units per cubic centimeter for Type I and 150 units per cubic
centimeter for Type II. Comparison should also be made of the per-
centage survival of other dilutions of the unknown and control serum.

e. *Records and reports.* Reports of tests shall be in the form of a
summary, as outlined on page 661.

MASSACHUSETTS ANTITOXIN AND VACCINE LABORATORY

The method is a modification of the Park-Cooper technique[1054] and
of the American Drug Manufacturers' Association test as revised De-
cember 29, 1933. The method herein described has been in routine use
at the Massachusetts Antitoxin and Vaccine Laboratory since February,
1933.

a. *Culture.* Virulence. The virulence of the cultures used should be
such that no more than 3 to 10 organisms, as determined by 24-hour
blood-agar plate counts, are required to kill at least two of three mice
within 48 hours after intraperitoneal inoculation. Passages of the cul-
tures through mice are made five days each week for maintenance of
virulence.

The culture used for a protection test is obtained in the following
manner: A mouse is injected intraperitoneally with 0.5 cc. of an 18-hour
broth culture; 6 hours later the mouse is chloroformed and a sample of
the heart's blood is inoculated, by means of a sterile capillary pipette,
into broth and on a blood-agar plate. After 18 hours growth, if the
cultures are pure, a transfer of 1.0 cc. of the broth culture is made to
15.0 cc. of broth and a drop of defibrinated normal horse blood added.
This culture is allowed to grow for 4½ hours and is the one used in the
test.

(1) Virulence Test: As a part of the mouse protection test, the

virulence of the culture is checked by injecting three doses into mice in triplicate with 0.5 cc. of dilutions of the 4½ hour culture made in peptone solution. Fluctuations in the virulence and amount of growth of the culture from time to time may require variations in the dilutions, and those used in a given test are determined upon the basis of previous tests. The dilutions employed have thus varied from 1:100 million, 1:300 million, and 1:1,000 million to 1:250 million, 1:750 million, and 1:2,500 million for Type I; and 1:90 million, 1:270 million, and 1:900 million to 1:125 million, 1:375 million, and 1:1,250 million for Type II. Dilutions for other types are determined by experience. Virulence test dilutions are made in sterile test tubes.

(2) Test Dose of Culture: The test dose of culture used in determining the protective power of serums is 0.5 cc. of a dilution of the 4½ hour culture made in peptone solution. The dilution employed depends upon fluctuations in growth and virulence (mentioned above) and must be determined by experience. For Type I tests, the dilution used has varied from 1:1,000 to 1:2,500; for Type II from 1:9,000 to 1:12,500. The test dilution of culture is usually made in a sterile 125 cc. or 250 cc. Erlenmeyer flask, the amount depending upon the number of mice to be injected. A loopful of the test dilution is streaked on a blood-agar plate as a purity test and, in addition, three mice are injected with the test dose of culture alone.

Illustrations of the method of making culture dilutions in typical tests, for a total of 132 mice, are shown in the following tables:

TYPE I

Tube No.	cc. of culture	cc. of sterile peptone	Dilution	cc. contained in each 0.5 cc. of original culture
1	1	4	1:5	1×10^{-1}
2	1 of No. 1	9	1:50	1×10^{-2}
3	2 of No. 2	18	1:500	1×10^{-3}
4	15 of No. 3	60	1:2500*	2×10^{-4}
5	1 of No. 4	9	1:25,000	2×10^{-5}
6	1 of No. 5	9	1:250,000	2×10^{-6}
7	1 of No. 6	9	1:2,500,000	2×10^{-7}
8	1 of No. 7	9	1:25,000,000	2×10^{-8}
9	1 of No. 8	9	1:250,000,000	2×10^{-9}
10	2 of No. 9	4	1:750,000,000	0.66×10^{-9}
11	1 of No. 9	9	1:2,500,000,000	0.2×10^{-9}

* This is the test dilution of culture.

TYPE II

1	1	9	1:10	5×10^{-2}
2	2 of No. 1	3	1:25	2×10^{-2}
3	1 of No. 2	9	1:250	2×10^{-3}
4	2 of No. 3	18	1:2500	2×10^{-4}
5	15 of No. 4	60	1:12,500*	4×10^{-5}
6	1 of No. 5	9	1:125,000	4×10^{-6}
7	1 of No. 6	9	1:1,250,000	4×10^{-7}
8	1 of No. 7	9	1:12,500,000	4×10^{-8}
9	1 of No. 8	9	1:125,000,000	4×10^{-9}
10	2 of No. 9	4	1:375,000,000	1.33×10^{-9}
11	1 of No. 9	9	1:1,250,000,000	0.4×10^{-9}

* This is the test dilution of culture.

b. *Serums*. All dilutions of serums are made in physiological salt solution, the amounts of diluent being drawn from an accurately graduated burette. Accurately graduated pipettes are used for mixing dilutions, using a separate pipette for each mixture. Each dilution is drawn up into the pipette 25 times to ensure thorough mixing. Excess serum adhering to the outside of the pipette after removal from the original sample is removed with a pledget of cotton. Not less than 1.0 cc. of a given serum dilution is used for making any higher dilution, and the volume of each dilution is kept as small as possible (usually 15 cc. or less) so that mixing may be thorough. (In making the first dilution of the control serum only 0.5 cc. of serum is used in order to conserve it.) The test dose of all serum dilutions is contained in a volume of 0.5 cc.

(1) Control Serum: The control serum is one supplied by the National Institute of Health and is, at present, P 11 to which have been assigned unit values of 300 and 150 units per cubic centimeter for Types I and II respectively. The dilutions used are for Type I, 1:1,000, 1:2,000, and 1:4,000; for Type II, 1:1,250, 1:2,500, and 1:5,000. These dilutions are made in the following manner:

TYPE I

0.5 cc. serum + 4.5 cc. saline = 1:10 (a)
1.0 cc. of (a) + 9 cc. saline = 1:100 (b)
2.0 cc. of (b) + 8 cc. saline = 1:500 (c)
8.0 cc. of (c) + 8 cc. saline = 1:1000 (d)
4.0 cc. of (d) + 4 cc. saline = 1:2000 (e)
2.0 cc. of (d) + 6 cc. saline = 1:4000 (f)

TYPE II

(a), (b), and (c) dilutions as above.
6.0 cc. of (c) + 9 cc. saline = 1:1250 (d)
4.0 cc. of (d) + 4 cc. saline = 1:2500 (e)
2.0 cc. of (d) + 6 cc. saline = 1:5000 (f)

In each case, only the (d), (e), and (f) dilutions are used for injecting mice.

(2) Unknown Serums: The dilutions used for injecting mice must bear the same ratio to each other as dilutions (d), (e), and (f) of the control serum. As concentrated serums are ordinarily much stronger than the control, obviously the dilutions will be much higher. For example, if a serum being tested for Type I antibody is assumed to be about 10 times as strong as the control serum, the dilutions used for injecting mice will be 1:10,000, 1:20,000, and 1:40,000, these being reached by making dilutions in steps similar to those for the control.

c. *The test.* At least two complete tests giving consistent results and preferably made on different days, using 10 mice for each dilution of serum, must be made for final estimation of the potency of an unknown serum.

(1) The Mice: It is preferable to use mice of a highly inbred, uniformly susceptible stock, in sound, healthy condition. The weights should lie between 16 and 20 grams each.

(2) Method of Injection: Serum and culture dilutions are not mixed prior to injection. Two 2 cc. vaccine syringes, fitted with 26-gauge, ⅜ inch Schick needles, are used for injecting the culture. Two ordinary 2 cc. hypodermic syringes, fitted with needles as above, are used for each serum. One operator fills the syringes, a second makes the injections, and an assistant handles the mice. The serums are always injected first, starting with the highest dilution, and followed immediately by the test dose of culture. Any mice improperly injected are discarded and replaced by others. Four serums (including the standard) and the requisite controls require 132 mice. From 30 to 40 minutes are required for injecting this number.

(3) Length of Test: Observations are made at least twice each day and the approximate hour of death is recorded for mice found dead. The test is terminated at the end of 96 hours and all mice living at this time are recorded as survivals.

(4) Necropsies: Necropsies should be performed on a sufficient

number of mice to prove death due to uncontaminated Pneumococcus infection. Streaking a sample of the heart's blood from a sterile capillary pipette on a blood-agar plate is adequate.

d. *Interpretation of the test.* In interpreting the test, no attempt is made to identify an end-point dilution, because such a procedure is statistically unsound inasmuch as it considers the fate of too small a group (10 mice). As the same number of mice is used for each serum and as the doses used for each serum are in the same ratio to each other, it is permissible to take into account the whole number (30) injected with each serum. The method of computation of potency values is, therefore, based on the equation:

$$\text{Units per cc. in unknown serum} = \frac{x}{y}\left(a + \frac{a\,(s - s')}{n}\right)$$

x = largest amount of control serum injected
y = largest amount of unknown serum injected
a = units per cc. in control serum
s = number of mice surviving on unknown serum
s′ = number of mice surviving on control serum, and
n = total number of mice injected with all dilutions of any one serum (see above).

If s is less than s′, then the part of the formula within the inner parentheses has a minus value and is subtracted from a, not added to it. The value of n will be 30, 60, 90, etc., depending upon the number of tests from which calculations are made.

The form of protocol used for reporting to the National Institute of Health does not show the value for x and y. The formula can be applied to these protocols if the following definitions are used for these values:

x = the smallest number of units tested for with unknown serum, and
y = smallest number of units tested for with control serum.

The application of the formula to the interpretation of the CP 77 B Types I and II protocols (pages 659 and 660 respectively) summarized on the attached National Institute of Health protocol (page 661), using the second definitions for x and y, is: x = 1500, y = 150, a = 300, s = 14, s′ = 15, n = 30; so

$$\frac{x}{y}\left(a + \frac{a\,(s - s')}{n}\right) = \frac{1500}{150}\left(300 + \frac{300\,(14 - 15)}{30}\right) = 2900 \text{ Type I units per cc., and}$$

$$= \frac{750}{75}\left(150 + \frac{150\,(15 - 9)}{30}\right) = 1800 \text{ Type II units per cc.}$$

e. *Protocols and records.* Protocols of each protection test are kept in loose leaf notebooks for a year, after which they are filed in fireproof drawers. Samples of protocols of Type I and Type II tests appear on pages 659 and 660, and on page 661 there is a sample of the protocol for reporting to the National Institute of Health. The potencies of the various lots of serum for distribution are recorded on individual lot record cards.

Note: The method of potency testing has been modified to the extent that five dilutions of each serum are used instead of three, although the range of dilutions remains the same; and the number of mice injected with each dilution is six instead of ten. For example, in place of ten mice on each of the dilutions 1:1,000, 1:2,000, and 1:4,000 there are six mice on each of the dilutions 1:1,000, 1:1,500, 1:2,000, 1:3,000, and 1:4,000.

Furthermore, the method of interpretation described on page 657 has certain inherent faults which occasionally lead to an incorrect result. Therefore, we have been using since March 1939 the method described by Reed.* A simplification of this method is described by Reed and Muench.† Both depend upon accumulating, with the deaths and survivals at the given dilutions, the deaths at lower serum dilutions and the survivals at higher serum dilutions. These methods are probably increased in reliability when a reasonably large number of animals is used and when at least two points fall fairly close to, and on either side of, the 50 per cent end-point.

<div align="right">

E. S. R.

L. A. B.
</div>

March, 1941

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* Reed, Lowell J. Chapter II. *In* Biological effects of radiation. Edited by B. J. Duggar. New York: McGraw-Hill Book Co., Inc., 1936.

† Reed, Lowell J., and Muench, N. A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 27 (3):493, May 1938.

Antipneumococcic Serum Potency Test - Protection Method - Type **II**

By **L. A. B. & E. C. W.** Date **12 - 8 - 36**

Date test begun **12 - 8 - 36** Date test finished **12-12-36**
Mouse weights vary from **16** to **20** grams
Volume of test dilutione of serums injected **0·5 cc.**
Test dilution of culture **1 : 11,000** Volume injected **0·5 cc.**
Number organisms in test dose **80,000** Number lethal doses in test dose **80,000**

Serum Control No. **P 11.**
Serum Dilutions

	1:1250	1:2500	1:5000	
Dead at	**43 hrs.**	**19**	**19**	:
	67	**24**	**19**	: **1 : 11,000**
	91	**43**	**19**	: Culture Control:
	S	**43**	**19**	: Plate: OK·
	S	**43**	**24**	: Result: Dead at
	S	**43**	**24**	: **19 hrs.**
	S	**48**	**24**	: **19**
	S	**48**	**43**	: **24**
	S	**S**	**43**	:
	S	**S**	**43**	:

Serum Lab. No. **CP 11 B**
Serum Dilutions

	1:12,500	1:25,000	1:50,000
Dead at	**91 hrs.**	**19**	**24**
	S	**43**	**24**
	S	**67**	**43**
	S	**67**	**43**
	S	**91**	**43**
	S	**S**	**43**
	S	**S**	**48**
	S	**S**	**67**
	S	**S**	**72**
	S	**S**	**S**

Pneumococcus Culture Controls
Broth used **11-18-36** Seed culture used **PN 11** Min-12 Volume seed used **1.0 cc.**
Vol. broth inoculated **15.0 cc.** No. hours grown **4 1/2** Turbidity **Good**
Virulence Test
0.5 cc. of following dilutions injected:
1 : 110 million 1 : 330 million 1 : 1100 million

No. orgs. inject.:	**8**	**1**	**± 1**
RESULT: DEAD AT.	**24 hrs.**	**19**	**24**
	24	**24**	**S**
	43	**S**	**S**

Plate Counts
0.5 cc. of following dilutions plated:
1 : 110 million 1 : 330 million 1 : 1100 million

Plate 1 **11** **2** **1**
Plate 2 **5** **0** **0**
Serum dilutions from **10:55** to **11 : 14** Culture dilutions from **1:40** to **2:10**
Injections started **2:15** Injections finished **2:50**
Interpretation of Test

CP 11 B contains about 1800 Type II units per cc

SAMPLE PROTOCOL OF A TYPE II TEST, MASSACHUSETTS
DEPARTMENT OF PUBLIC HEALTH

Antipneumococcic Serum Potency Test - Protection Method - Type I

By L.A.B & E.C.W.   Date   12-2-36

Date test begun   12-2-36   Date test finished   12-6-36
Mouse weights vary from 16 to 20 grams
Volume of test dilutions of serums injected 0.5 cc.
Test dilution of culture 1:2500   Volume injected 0.5 cc.
Number organisms in test dose 400,000 Number lethal doses in test dose 100,000

Serum Control No. P.11
Serum Dilutions

	1:1000	1:2000	1:4000	
Dead at	67 hrs.	67	43	:
	67	67	43	: 1:2500
	S	67	43	: Culture Control:
	S	92	43	: Plate: OK
	S	S	43	: Result: Dead at
	S	S	48	: 43 hrs.
	S	S	48	: 43
	S	S	67	: 67
	S	S	92	:
	S	S	S	:

Serum Lab. No. CP 11B
Serum Dilutions

	1:10,000	1:20,000	1:40,000
Dead at	43 hrs.	67	24
	67	67	43
	S	69	43
	S	92	48
	S	S	67
	S	S	67
	S	S	67
	S	67
	S	S	67
	S	S	92

Pneumococcus Culture Controls
Broth used 11-18-36 Seed culture used Pn I₃ Mi₁₁·₁·₃₀ Volume seed used 1:0 cc
Vol. broth inoculated 15.0 cc. No. hours grown 4½ Turbidity good
Virulence Test
0.5 cc. of following dilutions injected:

	1:250 million	1:750 million	1:2500 million
No. orgs. inject.:	4	1	±1
Result Dead At	43 hrs.	S	S
	43	S	S
	67	S	S

Plate Counts
0.5 cc. of following dilutions plated:

	1:250 million	1:750 million	1:2500 million
Plate 1	3	1	1
Plate 2	5	1	0

Serum dilutions from 11:44 to 12:44   Culture dilutions from 1:56 to 2:22
Injections started 2:38   Injections finished 3:18
Interpretation of Test

CP 11B contains about 2900 Type I units per cc.

SAMPLE PROTOCOL OF A TYPE I TEST, MASSACHUSETTS
DEPARTMENT OF PUBLIC HEALTH

Summary of Pneumococcus Types I and II Potency Tests

Product _____
(Nat. Inst. of Health Code No.)

Date of Test	Dose of Culture used 0.5 cc. of	Type I						Type II					
		Control Serum P-11						Control Serum P-11					
		150 U		300 U		600 U		15 U		150 U		300 U	
		S	D	S	D	S	D	S	D	S	D	S	D
12-2-36	1:2500	8	2	6	4	1	9						
12-8-36	1:11,000							7	3	2	8	0	10
Total		8	2	6	4	1	9	7	3	2	8	0	10
		Test Serum ≠ CP 77B						Test Serum ≠ CP 77B					
		1500 U		3000 U		6000 U		750 U		1500 U		3000 U	
		S	D	S	D	S	D	S	D	S	D	S	D
12-2-36	1:2500	8	2	6	4	0	10						
12-8-36	1:11,000							9	1	5	5	1	9
Total		8	2	6	4	0	10	9	1	5	5	1	9

From this summary the Potency of Lot ≠ CP 77B is estimated to be for

Type I   2900   units per cc.

Type II   1800   units per cc.

Product - Concentrated Antipneumococcic Serum

Type   - I and II

Lot No. - CP 77B   Name of Producing Laboratory

Nat. Inst. of Health Code No.   Signed _____

                                     Date _____

SAMPLE PROTOCOL OF TYPE I AND TYPE II TESTS, FOR REPORTING
TO THE NATIONAL INSTITUTE OF HEALTH

VIII. Sterility Tests on Antipneumococcic Serum

BULK STERILITY TESTS (National Institute of Health Regulations)

For sterility tests, from each bulk container holding more than 1 liter at least 10 cc. should be planted into broth fermentation tubes; for bulk containers of less than 1 liter capacity at least 3 cc. should be planted into enough fermentation tubes so that the preservative shall not exceed 0.01 per cent of the broth used. If the bulk containers are opened at any subsequent manipulation, two 5 drop and two 20 drop fermentation tubes should be planted from each container. If contaminations appear in any of the bulk tests, the test may be repeated, but no lot shall be passed until such tests show no growth, and if the same contaminating organism appears in more than one test, the material shall be discarded or resterilized.

Inoculated tubes are incubated at 37° for seven days and should be examined on the second, fourth, and seventh days after planting, and agitated only after 48 hours incubation to ensure initial anaerobiosis; the lowest part of the bend should be examined for slight growths, as well as both arms. In case the material is turbid, transplants to fresh fermentation tubes, as well as smears, should be made on the seventh day.

STERILITY TESTS ON FINAL CONTAINERS (National Institute of Health Regulations)

Of the final containers selected at random from each lot, three to ten shall be tested in accordance with the following schedule:

Total Number of Containers in the Lot	Number of Containers to be Tested
100 or less	3
101 to 150	4
151 to 200	5
201 to 250	6
251 to 300	7
301 to 350	8
351 to 400	9
Over 400	10

At least one 5 drop and one 20 drop fermentation tube shall be planted from each container. In the case of products to be administered in doses larger than 1 cc., the entire dose up to 5 cc. from each container tested shall be planted in fermentation tubes containing enough

broth to dilute the preservative so that its concentration shall not exceed 0.01 per cent, but not more than 5 cc. need be planted from any container. In case contaminations appear in any of the tubes planted, the test may be repeated with the same number of containers but no lot shall be passed until the final test shows no growth throughout, and a lot shall be discarded if the same organism appears in more than one test.

IX. Preparation of Diagnostic Antipneumococcic Rabbit Serum

MASSACHUSETTS ANTITOXIN AND VACCINE LABORATORY METHOD

a. *Vaccine.* Eighteen hour broth cultures of highly virulent, type-specific pneumococci are centrifuged, the sediment taken up in physiological salt solution in quantities to give a corrected Gates reading of 2.0, and the suspension heated for 15 minutes at 100°. This results in a suspension containing approximately 25 billion organisms per cubic centimeter and is used as a stock supply of vaccine. No preservative is added. For the injection of rabbits, the stock vaccine is diluted 1 to 10 with physiological saline solution, thus giving a suspension containing about 2.5 billion organisms per cubic centimeter.

b. *Injection of rabbits.* A minimal number of six rabbits should be maintained on each type of vaccine. All injections are given intravenously, the schedule and dosages following the scheme given below:

Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1	0.1	0.2	0.5			
2			0.2	0.5	1.0	
3	REST					
4	0.5	1.0	2.0			
5			1.0	2.0	3.0	
6	REST					
7	1.0	2.0	3.0			
8			1.0	2.0	3.0	
9	REST					
10					BLEED	
11	1.0	2.0	3.0			
12			1.0	2.0	3.0	
13	REST					
14					BLEED	

c. *Bleeding of rabbits.* The rabbits are bled by cardiac puncture every three weeks after routine bleedings are initiated and, with careful handling, the animals may be kept for an average of at least a year.

It has been found advisable to have available a supply of buffered glucose solution to administer intravenously to the occasional animal suffering severe systemic shock after having been bled. A minimum of 20 cc. of blood is removed from each rabbit and placed in 50 cc. centrifuge tubes, and slanted until coagulation has occurred. After standing for a few hours at room temperature, the clots are broken, and the tubes of blood stored overnight at 4°. The next morning the serum is drawn off, centrifuged, pooled, and preserved with 0.3 per cent (final concentration) of tricresol.

d. *Testing of serum.* The pooled serum of each type is tested for type-specific Neufeld reactions and for heterologous reactions with known closely related types. For these tests, mice are injected intraperitoneally with cultures of the types of Pneumococcus desired, and the peritoneal exudate washed out with saline solution about six hours after inoculation. Neufeld tests are set up in the usual manner, and if satisfactory results are obtained and no cross-reactions observed, the serum may be either filled immediately into separate containers, or added to the stock supply of serum of the respective types.

e. *Dilution of serum.* It is possible, after high titered serums are obtained, to obtain satisfactory Neufeld reactions with type-specific serums diluted with normal rabbit serum. The degree of dilution must be determined by tests, taking into consideration the amount of dilution which may occur in making the pools used in routine type determination in the diagnostic laboratory.

NOTE: Since diagnostic serums have been made subject to Federal license, the method of testing prescribed by the National Institute of Health (Minimum Requirements for Pneumococcus Typing Serum, Jan. 17, 1939) has superseded that described in paragraph "d" above. The method is also described by Dr. Bernice E. Eddy, Public Health Reports, 55 (9):347, Mar. 1, 1940.

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30, 32, 34, 35, 36, 46, 47, 627, 628

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INDEX

Monkey(s) (*cont.*):
  experiments on, 481–483
  in tests for chill-producing proper-
    ties of serum, 564–565
  susceptibility to Pneumococcus
    as carrier, 192
    Cebus capucinus, 192, 193
    Ceropithecus callitrichus, 192
    epidemics in, 191, 483–484
    inoculation, 191–193, 577
    lobar pneumonia in, 192, 193
    Macacus cynomolgos, 193, 368
    Macacus rhesus, 192, 193
    Pithecus philippinensis, 192
    variations, individual, 193
Morphology of Pneumococcus, 30–35
Mouse (mice)
  immunizing action of polysaccha-
    rides, 255
  in isolation of Pneumococcus, 35,
    626–630
  in potency tests on antipneumococ-
    cic serum, 407–408, 567–576, 653–
    661
  in protection tests, host response,
    420, 572, 574
  in safety tests for antipneumococcic
    serum, 588–589, 591
  in serological classification, 104,
    105, 106, 108, 111, 113, 114
  in tests for preservatives, 588
  in therapeutic tests on antipneumo-
    coccic serum, 578–579
  in type determination, 120–122, 628–
    630, 631
  protective action of enzyme in, 305–
    306
  purpura in, 87, 89, 90
  susceptibility to Pneumococcus
    age, 189
    as carrier, 189
    Bacillus typhi murium, 190
    body-weight, 189, 190
    breed, 189
    dose of Pneumococcus necessary
      to infect, 188
    effect of alcohol, 190
    effect of toxins, 97, 99

Mouse (mice) (*cont.*):
  susceptibility to Pneumococcus
    (*cont.*):
    inoculation, 188–190
    mixed infection, 188
    monocytes, 190
    pigmentation, 187, 188
    variation in response to infection,
      187, 190, 420, 572, 574
    white cells, 190
Mucin
  in blood, 240
  in capsule, 3, 7, 33, 239
Mucin, gastric, virulence of Pneumo-
  coccus enhanced by, 202
Mutation
  in serological type, 156–169
  in species, 169–177
Mycobacteriaceae, 320

National Institute of Health, potency
  tests, 567, 592, 648–650
Natural immunity. *See* Immunity to
  Pneumococcus
Necrotin, 92
Necrotizing principle of Pneumococcus
  in autolysates, 92–93, 98–99
  relation to hemotoxin, 92
Nephritis, Pneumococcus in, 16, 17
Neufeld *Quellung* phenomenon. *See*
  Quellung phenomenon
Nitrogen determination, precipitation
  tests, 643–644
Normal individuals
  opsonins in, 395–397
  Pneumococcus in, 215–216
  See also Carriers of Pneumococcus
Nucleoprotein(s) of Pneumococcus.
  See Protein fraction(s)
Nutrient media. *See* Media

Oliver's method of type determina-
  tion, 126
Olmstead's serological groups, 108–
  109
Ophthalmic reactions
  capsular polysaccharides in, 457–458
  in rabbit, 457

Ophthalmic reactions (*cont.*):
  nucleoprotein in, 457
  specificity, 458
Opsonins
  agglutinins, relation to, 431
  antiopsonins, 398–401
  antiphagins, 398
  Impedin, 399
  in pneumonia, 401–402, 438, 439
  method for demonstrating, 429, 431
  normal, 393, 395–397
  sensitization of pneumococci, 391–
    395
  See also Phagocytosis
Optical rotation of polysaccharides,
    246, 254, 255, 257, 273, 274, 281,
    291, 292, 293
Optochin, 512–520
  adjuvant action with immune se-
    rum, 518
  base, 512, 516, 518
  drug-fastness, 519–520
  effect in dissociation, 144–145
  effect on virulence, 519
  germicidal action, 513, 514, 515
  heightened susceptibility of Pneu-
    mococcus to, 520
  specificity, 520
  toxicity, 517
Oral administration of antigens, 348–
    351
Orchitis, Pneumococcus in, 226
Osteomyelitis, Pneumococcus in, 226
Otitis, Pneumococcus in, 20, 226, 227
Oxidation and reduction, 71–82
  analysis of reaction, 80–81
  B. acidophilus, 75
  B. bulgaricus, 75
  diphtheria bacilli, 77
  effect of catalase, 78
  hydrogen peroxide, 44, 74–82
  methemoglobin, 71–74
  methylene blue, 77
  oxidases, 71, 75
  peroxide, 71, 74–82
  reversibility of action, 81
  staphylococci, 77
  streptococci, 77

Oxidation and reduction (*cont.*):
  S. haemolyticus, 75
  S. viridans, 75
  xanthoprotein, 75

Panophthalmia, Pneumococcus in, 227
Parotitis, Pneumococcus in, 19, 226
Passive immunity. *See* Immunity,
    passive
Passive sensitization. *See* Sensitiza-
    tion, passive
Pathogenicity of Pneumococcus
  for experimental animals
    birds, 196
    cats, 193
    dogs, 7, 10, 16, 193–195
    guinea pigs, 7, 185–187
    horses, 195–196
    mice, 7, 187–190
    monkeys, 191–193
    rabbits, 7, 179–185
    rats, 190–191
  for man, 7, 214–237, 435
  See also Virulence
Peptonase, in Pneumococcus, 66
Peptone
  effect on growth, 38
  effect on hemolysis, 84
Pericarditis, Pneumococcus in, 8, 9,
    16, 226
Peritonsillar abscess, primary, Pneu-
    mococcus in, 20
Peroxidase in peroxide formation, 76
Peroxide formation
  by *B. acidophilus,* 75
  by *B. bulgaricus,* 75
  by Pneumococcus, 44, 74–82
  by *S. aureus,* 76
  by *S. haemolyticus,* 75, 76
  by *S. mucosus,* 76
  by *S. viridans,* 75
  effect of bacteria, 78
  effect of blood, 78
  effect of catalase, 78
  effect of cell washings, 77
  effect of gelatin, 77
  effect of glucose, 77
  effect of lactic acid, 77